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# **Staphylococcal fibronectin-binding proteins**

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**Thesis submitted for examination through the Open University**

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## Glossary of terms.

<i>agr</i>	accessory global regulator
Bbp	bone sialoprotein-binding protein
BSA	bovine serum albumin
CC	clonal complex
cfu	colony forming units
ClfA	clumping factor A
ClfB	clumping factor B
Cna	collagen-binding protein
Coa	coagulase
Cps	capsular polysaccharide
DIG	digoxigenin
DNA	deoxyribonucleic acid
EbpS	elastin-binding protein S
Efb	extracellular fibrinogen-binding protein
EMRSA	epidemic methicillin-resistant <i>S. aureus</i>
Eta & Etb	exfoliative toxins A and B
<i>fnb</i>	gene encoding fibronectin-binding protein
<i>fnbA</i>	gene encoding fibronectin-binding protein A
<i>fnbB</i>	gene encoding fibronectin-binding protein B
FnBP	fibronectin-binding protein
FnBPA	fibronectin-binding protein A
FnBPB	fibronectin-binding protein B
FTC-casein	fluorescein thiolcarbamoyl-casein derivative
Hlg	gamma toxin
Hsp	heat shock protein
HUVEC	human umbilical vein endothelial cells
ica	intercellular adhesin
kDa	kilodalton
LB	Luria-Bertani broth
Map	MHC class II analog protein
MSLT	multilocus sequence typing
M199	medium M199
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PFNBA4	multicopy plasmid carrying <i>fnbA</i>
PFNB4	multicopy plasmid carrying <i>fnbB</i>
Pvl	Panton-Valentine leukocidin
rFNBD	recombinant form of the binding region of fibronectin-binding protein B of <i>Streptococcus dysgalactiae</i>
SEA-J	staphylococcal enterotoxins A to J
SdrC, D & E	Sdr repeat proteins C, D and E
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
Spa	protein A
TBST	10mM Tris-HCl, 500mM NaCl, 0.1% Tween
THB	Todd Hewitt broth
TEM	transmission electron microscopy
TSA	trypticase soy agar
TSB	trypticase soy broth
TSST-1	toxin shock syndrome toxin 1
vWF	von Willibrand factor

## **Publications arising from this thesis.**

**Peacock SJ**, Foster TJ, Cameron B, and Berendt AR. The dominant pathway for *S. aureus* adherence to human endothelial cells *in vitro* is mediated by fibronectin-binding protein and endothelial cell surface fibronectin. *Microbiol* 1999;145:3477-3486.

**Peacock SJ**, Lina G, Etienne J, and Foster TJ. *Staphylococcus schleiferi* subsp. *schleiferi* expresses a fibronectin-binding protein. *Infect Immun* 1999;67:4272-4275.

**Peacock SJ**, Day NPJ, Thomas MG, Berendt AR., and Foster TJ. Clinical isolates of *Staphylococcus aureus* exhibit diversity in *fnb* genes and adhesion to human fibronectin. *J Infect* 2000;41:23-31.

Massey RC, Kantzanou MN, Fowler T, Day NPJ, Schofield K, Wann ER, Berendt AR, Höök M and **Peacock SJ**. Fibronectin-binding protein A of *Staphylococcus aureus* has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells. *Cellular Microbiol* 2001;3:839-851.

**Peacock SJ**, Moore CE, Justice A, Kantzanou M, Story L, Mackie K, O'Neil G, and Day NPJ. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. *Infect Immun* 2002;70:4987-4996.

## Abstract.

This thesis describes a series of studies examining the fibronectin-binding proteins (FnBPs) of the pathogenic bacteria *Staphylococcus aureus* and *Staphylococcus schleiferi*. The first two results chapters explore the role of FnBPs in the interaction between *S. aureus* and human endothelial cells *in vitro*. The impetus for studying this area was the likely importance of this interaction *in vivo* during the process of bacterial seeding from the bloodstream to distant sites, a common accompaniment to bacteraemia and invasive disease. Having demonstrated a central role for FnBPs in adherence to, and invasion of endothelial cells, the third results chapter describes phenotypic and genotypic variation in *fnb* genes and the proteins they encode in a large population of clinical *S. aureus* isolates. The fourth results chapter examines whether proteases, and in particular serine (V8) protease, influence *S. aureus* FnBP function, potentially modelling the bacterial cell surface and controlling the presence or absence of a functional adhesin. The fifth results chapter demonstrates that *S. schleiferi*, a coagulase-negative staphylococcus and a nosocomial pathogen, expresses a FnBP. In the final results chapter, the presence of *fnbA* encoding *S. aureus* FnBPA is compared between isolates associated with carriage and invasive disease, together with 32 other bacterial factors. The aims of this study were to identify virulence-associated genes (one of which was *fnbA*); to assess the cumulative effect of virulence-associated genes on virulence; and to identify gene combinations and determine if some combinations have a greater pathogenic potential than others.

## **Chapter 1. Introduction.**

### **1.1 General background.**

#### **1.1.1 The genus *Staphylococcus*.**

Staphylococci are gram-positive cocci of  $\sim 1\mu\text{m}$  in diameter which on direct gram-stained smears from clinical specimens may appear singly, in pairs or short chains but have a strong tendency to form clusters. Staphylococci grow under both aerobic and anaerobic conditions on blood agar and other non-selective media. Most staphylococcal species grow rapidly, forming colonies on solid agar of  $>1\text{-}2\text{mm}$  in diameter by 24 hours incubation. Colonies are usually smooth with a low convex profile and an entire edge, the colour of which varies between species and ranges from yellow pigmented (some strains of *Staphylococcus aureus*), through white to gray. Staphylococci are differentiated from streptococci by a positive catalase test in which hydrogen peroxide is converted into water and oxygen on contact with a colony, giving rise to the appearance of bubbles.

*S. aureus* is by far the most important pathogen among the staphylococci. This species is differentiated from others in the genus by a combination of tests, including mannitol fermentation, production of coagulase and deoxyribonuclease, and the expression of a cell wall-associated fibrinogen-binding protein (Collee *et al.*, 1996). Thirty-two species of coagulase-negative staphylococci (CoNS) have been described to date, of which 15 are indigenous to man (Kloos, 1997). Differentiation of CoNS species was originally based on a scheme devised by Kloos and Schleifer which relied on biochemical characteristics, while more recent species differentiation has used molecular methods based on DNA – DNA hybridization (reviewed in Kloos, 1997).

### 1.1.2 *S. aureus* carriage.

*S. aureus* is a common member of the normal human commensal flora. Colonization often occurs shortly after birth and appears to be a dynamic process. The umbilical stump is the first site to be colonized (Simpson *et al.*, 1960), but within a few days the reservoir of *S. aureus* shifts to the vestibule of the anterior nares (Simpson *et al.*, 1960). Other sites including the axilla, perineum and pharynx may also be colonized (Williams, 1963), but elimination of nasal carriage by topical antibiotics leads to loss of carriage elsewhere (Reagan *et al.*, 1991), indicating that the nares represent the dominant ecological niche. The epithelium in the anterior nares is stratified, keratinized, non-ciliated epithelium, setting it apart from the rest of the nasal cavity and respiratory tract which is lined with ciliated columnar epithelium. Some healthy children and adults carry *S. aureus* in the pharynx as detected by throat swab, but scanning electron microscopy using human nasal tissue obtained from surgical specimens has demonstrated that adherence of *S. aureus* to ciliated epithelium is poor (Shuter *et al.*, 1996). It is unclear, therefore, whether throat carriers are a subgroup of the population who can maintain *S. aureus* in this anatomical site, or whether organisms are temporary residents.

Longitudinal studies have reported that three carriage patterns can be distinguished in the healthy adult population, with approximately 20% of individuals being persistent *S. aureus* carriers, about 60% intermittent carriers, and 20% persistent non-carriers (reviewed in Kluytmans *et al.*, 1997). This is likely to be an over-simplification, not least because the duration of follow-up is often limited. In addition, comparison of results between studies is made difficult by the lack of conformity in the methods of ascertainment and carriage definitions as well as the absence of information on antibiotic exposure, an important confounding variable. Despite these reservations, there is sufficient data to support the idea that some individuals within the population are never,

or hardly ever positive for *S. aureus*, others are infrequent carriers, and the remainder are usually carriers.

### **1.1.3 The relationship between carriage and disease.**

Three strands of evidence support the view that nasal carriage of *S. aureus* and the development of staphylococcal infection are linked. First, rates of infection are higher in carriers than non-carriers. This has been demonstrated in a range of clinical settings studied to date, including the development of post-operative wound infections (Weinstein, 1959), in patients undergoing continuous ambulatory peritoneal dialysis and haemodialysis (Luzar *et al.*, 1990; Yu *et al.*, 1986), and in those infected with the human immunodeficiency virus (Weinke *et al.*, 1992). Second, studies comparing carriage and infecting isolates of *S. aureus* have demonstrated that individuals are usually infected with their own carriage isolate (Luzar *et al.*, 1990; Nguyen *et al.*, 1999; Yu *et al.*, 1986). This has recently been validated in a large study conducted by von Eiff *et al.* in which greater than 80% of isolates from *S. aureus* bacteraemic infections were identical to strains carried in the patients nares (von Eiff *et al.*, 2001). Third, the temporary eradication of carriage by the use of topical mupirocin has been shown to result in a reduction in nosocomial infection in dialysis patients and in those undergoing surgery (Boelaert *et al.*, 1993; Yu *et al.*, 1986; Perez-Fontan *et al.*, 1993; Kluytmans, 1998).

### **1.1.4 *S. aureus* infection in man.**

Considering the frequency of carriage in the population and the association between carriage and disease, attack rates for invasive *S. aureus* infection are comparatively low (estimated incidence 10/100,000 in Oxfordshire, personal communication Dr N. Day). Nonetheless, *S. aureus* is a major cause of community-acquired and nosocomial infection (Emori & Gaynes, 1993; Steinberg *et al.*, 1996). *S. aureus* is the second most common blood culture isolate in the

UK after *Escherichia coli* according to figures from the Public Health Laboratory Service, and is by far the commonest hospital-acquired infection (CDSC, 1998). It is also a common tropical pathogen; for example, it has recently been identified as the first or second commonest cause of community-acquired bacteraemia (depending on the year studied) in Vientiane, the capital city of Laos (Mahosot Hospital, personal communication, Dr Paul Newton).

Clinical manifestations of *S. aureus* infection are diverse, and include superficial infections (for example, carbuncles, furuncles, cellulitis, lymphangitis and lymphadenitis), bacteraemia, endocarditis, pneumonia, osteomyelitis, septic arthritis, post-operative wound infection, and infections of medical devices including intravenous devices, prosthetic joints and peritoneal dialysis catheters (Waldvogel, 2000). *S. aureus* also causes toxin-mediated disease through the production of epidermolytic toxins (the cause of staphylococcal scalded skin syndrome), enterotoxins (a cause of food poisoning), and toxic shock syndrome toxin 1 (Bohach *et al.*, 1997).

## **1.2. Determinants of *S. aureus* infection.**

### **1.2.1 Host factors.**

Nasal carriage is an important determinant of invasive disease, but host factors that are associated with carriage and/or invasive disease are poorly understood. Host genetic factors are known to be an important determinant of early death due to sepsis in general (Sorensen *et al.*, 1988), but there are no data in the published literature defining the existence of susceptibility loci in *S. aureus* disease. Specific immune defects such as chronic granulomatous disease and Job's syndrome are known to predispose individuals to infection with *S. aureus* (Donabedian & Gallin, 1983), but the pathways (and variations thereof) which influence whether disease develops in the normal host are not known.



### **1.2.2 Bacterial factors.**

Although the pathogenesis underlying many of the clinical manifestations of human *S. aureus* disease are poorly understood, it is widely held that bacterial factors including toxins, cell wall-associated adhesins and secreted exoproteins are involved in the disease process (Projan & Novick, 1997). The evidence for this is strongest in the case of toxin-mediated *S. aureus* diseases such as toxic shock syndrome, scalded skin syndrome and food poisoning (Bohach *et al.*, 1997). However, the vast majority of cases of severe *S. aureus* disease cannot be explained by the action of a single virulence determinant, and it is likely that a number of factors act in combination during the infective process. Support for this comes from animal models where outcome has been compared following infection with isogenic mutants deficient in a single putative virulence determinant, in which infection was attenuated but not prevented (Hienz *et al.*, 1996; Moreillon *et al.*, 1995). In addition, clinical isolates associated with human infection may be naturally deficient in a range of putative virulence determinants. It seems likely that *S. aureus* strains associated with human infection have variable combinations of pathogenic determinants. It is also possible that the presence or expression of given combinations varies depending on the type of infection and genetic susceptibility of the affected host. The capacity to examine multiple bacterial factors are only just emerging, and the current understanding of putative virulence determinants is mainly derived from the experimental evaluation of individual bacterial components.

### **1.2.3 *S. aureus* adhesins.**

A range of *S. aureus* cell wall-associated proteins have been characterised and shown to promote adherence to extracellular matrix proteins and/or soluble plasma components (Patti *et al.*, 1994a). Such adhesins may play a key role in colonization and invasive disease. The best characterised of these are protein A (Spa) (Uhlen *et al.*, 1984), collagen-binding protein Cna (Patti *et*

*al.*, 1992), the fibrinogen-binding proteins ClfA, ClfB and Efb (formally fib) (Boden & Flock, 1994; McDevitt *et al.*, 1994; Ni Eidhin *et al.*, 1998), and the two fibronectin-binding proteins FnBPA and FnBPB (Greene *et al.*, 1995; Jonsson *et al.*, 1991; Signas *et al.*, 1989). Additional staphylococcal proteins include three members of the serine-aspartate multigene family (SdrC, SdrD and SdrE) of unknown function (Josefsson *et al.*, 1998); the bone sialoprotein-binding protein Bbp, (Tung *et al.*, 2000); the elastin-binding protein EbpS (Park *et al.*, 1996); the major histocompatibility complex class II analog Map and the highly similar extracellular adhesin protein Eap (McGavin *et al.*, 1993a; Palma *et al.*, 1999); the plasmin-sensitive Pls (Savolainen *et al.*, 2001), and biofilm-associated protein (Bap) (Cucarella *et al.*, 2002).

With the exception of Efb, EbpS and Map/Eap, these proteins have features that are characteristic of Gram-positive bacterial surface-expressed proteins, including a secretory signal sequence at the N-terminus, and at the C-terminus a positively charged tail, a hydrophobic transmembrane domain and a wall-spanning region containing an LPXTG motif (Foster & Hook, 1998). The latter represents the target of a specific enzyme (sortase) which cleaves the motif between the threonine and glycine residues. The protein is then covalently anchored to the peptidoglycan cell wall (Mazmanian *et al.*, 1999; Schneewind *et al.*, 1995; Ton-That *et al.*, 1997).

### **1.3 *S. aureus* fibronectin-binding proteins.**

#### **1.3.1 Description of *S. aureus* fibronectin-binding proteins.**

*S. aureus* was first reported to bind fibronectin in 1978 (Kuusela, 1978); labelled fibronectin bound to the bacteria, while unlabelled Fn was inhibitory. Four years later Espersen and Clemmensen isolated a FnBP from sonicated *S. aureus* strain Newman using affinity chromatography (Espersen & Clemmensen, 1982). Two bands were identified with relative molecular masses of 197,000 and 60,000 kDa. A complex could be demonstrated between purified *S. aureus*

protein and fibronectin using crossed immunoelectrophoresis. Froman *et al* reproduced these data, and in addition reported that degradation of FnBP by V8 protease generated a large number of peptides that retained fibronectin binding activity. This was interpreted to mean that FnBP contained several binding sites for fibronectin (Froman *et al.*, 1987).

The majority of the early work defining *S. aureus* FnBPs was conducted by Martin Lindberg and colleagues, Sweden. A gene encoding a FnBP was identified from *S. aureus* 8325-4, genomic DNA from which had been cloned into *Escherichia coli* (Flock *et al.*, 1987). Although not an ideal cloning vector for a Gram-positive cell wall-anchored adhesin, the relevant clone was identified by defining secretion of a product into the periplasm of *E. coli* which inhibited the binding of radiolabelled fibronectin to *S. aureus* Cowan. The region encoding fibronectin-binding activity was identified by sub-cloning, and was reported to be in a region that was later termed the D (repeat) region. The nucleotide sequence of *S. aureus fnbA* was subsequently defined for *S. aureus* 8325-4 (Signas *et al.*, 1989). The entire gene sequence was annotated based on regions of repetition using computer analysis, defining from N-terminus to C-terminus, the A region, two B repeats (each of 30 amino acids), the C region and three D repeats of 38 amino acids plus a partial fourth (Signas *et al.*, 1989).

A second gene termed *fnbB* located 682bp downstream of *fnbA* in *S. aureus* 8325-4 was reported two years later (Jonsson *et al.*, 1991). The gene was sequenced, and the amino acid sequence deduced and compared to that for the deduced sequence of *fnbA*. The two genes showed sequence homology in large parts. In FnBPB a stretch of 66 amino acids downstream to the signal peptide had 75% identity with the corresponding region of FnBPA. At the C-terminal site another 394 amino acid stretch was almost identical in both gene products. This stretch contained the D repeats, the wall spanning region and the hydrophobic membrane spanning region. The D repeats in FnBPB were noted to be highly conserved.

The two *fnb* genes were further characterised by Professor T. Foster, Dublin. Insertion mutations were constructed in *fnbA* and *fnbB*. Single mutants did not show a significant reduction in adherence to immobilized fibronectin, but the double mutant was completely defective (Greene *et al.*, 1995).

### **1.3.2 Fibronectin.**

Fibronectin is a ubiquitous glycoprotein that exists in body fluids and the extracellular matrix (reviewed in Potts & Campbell, 1994). It plays a major role in many important physiological processes, promoting cell adhesion and affecting cell morphology, migration, differentiation and cytoskeletal organisation via interactions with integrins. A host ligand for FnBP, fibronectin was the first extracellular matrix protein shown to act as a substrate for microbial adhesion (Kuusela, 1978). Each subunit of fibronectin is a mosaic protein made up of repeating modules of three types (I, II, III). There are 12 type I repeats each around 45 amino acids long and clustered into 3 groups, two adjacent type II repeats and 15-17 type III repeats. The initial secreted form is a dimer of two subunits held together by a pair of disulphide bonds near their C-termini. Dimeric fibronectins are soluble molecules but become insoluble in extracellular matrix fibrils where they are further disulphide-bonded into high molecular weight polymers. The modular proteins often correspond to the exon structure of the gene, and are organised into functional domains that contain binding sites for extracellular matrix proteins such as collagen and thrombospondin, cell surface receptors such as integrins, circulating blood proteins such as fibrin, and glycosaminoglycans such as heparin and chondroitin sulphate.

### **1.3.3 Interactions between *S. aureus* FnBPs and fibronectin.**

Investigation of the interaction between fibronectin and *S. aureus* commenced at around the time that FnBPs were first identified. Binding was demonstrated to be non-covalent and saturable, and was dependent on time and fibronectin concentration (Maxe *et al.*, 1986; Proctor *et al.*, 1982). Adherence was affected by bacterial growth phase; *S. aureus* harvested in logarithmic growth phase had a higher number of binding sites compared with stationary phase organisms (Proctor *et al.*, 1982), and there was a decrease in adherence to immobilized fibronectin as the duration of incubation increased (Maxe *et al.*, 1986).

#### **1.3.3.1 Binding sites for FnBPs on fibronectin molecule.**

Using fibronectin fragments, *S. aureus* was found to adhere to both the NH<sub>2</sub> terminal (Kuusela *et al.*, 1984; Kuusela *et al.*, 1985; Mosher & Proctor, 1980; Sakata *et al.*, 1994; Bozzini *et al.*, 1992), and to a 120-140kDa COOH terminal fragment (Kuusela *et al.*, 1984; Kuusela *et al.*, 1985). Bacterial adherence to immobilized fibronectin was inhibited by the presence of soluble fibronectin or a 29kDa NH<sub>2</sub> terminal fragment, and by pre-incubating substrate with purified FnBP (Maxe *et al.*, 1986). The N-terminus of fibronectin is now recognised as the domain primarily recognised by FNBPs. This region contains five type I 'finger' modules. Investigation using recombinant truncated fibronectin molecules generated with deletions or mutations in the N-terminal type I modules suggested that binding of *S. aureus* required all five type I modules (Sottile *et al.*, 1991), evaluation using recombinant FnBP D region peptides indicating that binding occurs primarily to fingers 4 and 5 (Huff *et al.*, 1994).

### 1.3.3.2 Ligand binding sites on FnBP.

Sequencing of *fnbA* permitted the construction of synthetic FnBP peptides, an important step towards detailed analysis of FnBP-ligand binding. The original cloning experiments of *fnbA* suggested that binding to fibronectin occurred via the D repeat region. Three peptides mimicking the structure of each 38 amino acid unit of FnBPA were constructed, all of which were found to inhibit binding of soluble fibronectin (intact or 29kDa N-terminal fragment) to staphylococcal cells (Signas *et al.*, 1989). Synthetic peptides D1, D2 and D3 or D1-3 were also reported to inhibit the attachment of *S. aureus* to immobilized intact fibronectin or N-terminal 29kDa fragment (Raja *et al.*, 1990).

The region of the D peptide involved in fibronectin binding was then mapped using digested fragments of recombinant protein. An active peptide encompassing residues 15-36 of a 37 amino acid D3 synthetic peptide analog was isolated following trypsin digest. This had activity comparable with intact D3, suggesting that the FnBP binding region was within the C-terminal portion of the D repeat. Scrambling the amino acid sequence of this peptide or replacing the aspartic and glutamic residues resulted in loss of activity, while reduction in size from either the N- or C-terminal end resulted in peptides with greatly diminished activity (McGavin *et al.*, 1991). Synthetic peptides corresponding to the C-terminal 20 to 21 amino acids of each D repeat (D1, D2 and D3) demonstrated that each were independently capable of binding fibronectin, the D3 repeat binding with 5- to 10- fold greater affinity than either D1 or D2 (Huff *et al.*, 1994). The interaction between the D repeats and intact fibronectin or the N-terminal fragment is common to several Gram-positive bacteria able to recognise fibronectin, and recombinant ligand binding region proteins (equivalent to D repeat region) from *S. aureus*, *Streptococcus dysgalactiae* and *Streptococcus pyogenes* effectively inhibit the binding of each other to radiolabelled fibronectin (Joh *et al.*, 1994).

Further assays of chemically modified peptides and peptide fragments derived from chemical or proteolytic cleavage suggested that a core conserved sequence, defined as ED(T?S) (X9,10)GG(X3,4)(I/V)DF, within a 30-amino acid segment was present. ED(T?S) was reported to be non-essential, whereas the GG and the (I/V)DF together with acidic residues in the C-terminal half of the peptide were required for activity (McGavin *et al.*, 1993b).

The presence of additional FnBP binding sites was first suggested during cloning and sequencing of *fnbB*, when a sub-clone lacking the coding region for the D repeats was found to have fibronectin binding activity (Jonsson *et al.*, 1991). Evaluation of a series of truncates spanning FnBPA also demonstrated a second ligand binding region located outside the D repeat which appeared to be within the B region (Joh *et al.*, 1998). Using a phage display library of *S. aureus* genomic DNA constructed and panned against immobilized fibronectin and cultured osteoblasts, binding activity has also been located to the A region (Williams *et al.*, 2002). Thus, FnBP appears to have the ability to interact with fibronectin at several sites throughout the length of the protein.

#### **1.3.3.3 Interaction between FnBPs and fibronectin.**

Biophysical characterisation of recombinant FnBP suggested that this protein had little or no regular secondary structure in phosphate buffer at neutral pH. Binding to fibronectin led to the induction of a predominantly  $\beta$ -sheet structure in the recombinant adhesin (House-Pompeo *et al.*, 1996; Speziale *et al.*, 1996). Nuclear magnetic resonance techniques were used to study conformational properties of D1-D4; these provided further evidence that this protein was largely unstructured in aqueous solution at pH 6 (Penkett *et al.*, 1998; Penkett *et al.*, 1997). Examination of the interaction between D3 and D1-4 of FnBPA with a fibronectin module pair ( $^4\text{F1}^5\text{F1}$ ) using heteronuclear NMR spectroscopy showed that part of D3 converted from a disordered to a more

ordered, extended conformation on binding to  $^4\text{F1}^5\text{F1}$ . D1-4 also appeared to go from a disordered to a more ordered conformation (Penkett *et al.*, 2000).

#### 1.3.4 FnBPs and other ligands.

The recombinant form of the A region of FnBPA has also been shown to recognise fibrinogen (Wann *et al.*, 2000). Binding occurred to the gamma chain of fibrinogen, the chain recognised by the *S. aureus* fibrinogen-binding protein ClfA. Recombinant FnBPA competed with ClfA for binding to both immobilised and soluble fibrinogen, and over-expression of FnBPA in a bacterial mutant defective in ClfA and ClfB led to its adherence to soluble fibrinogen.

#### 1.3.5 Regulation of FnBPs.

*S. aureus* is known to have at least two major regulatory systems, termed the accessory global regulator (Agr) and staphylococcal accessory regulator (Sar). Both have been examined in relation to *fnb* expression. *agr* mutants were found to bind more fibronectin than wild type (Abdelnour *et al.*, 1993; Cheung *et al.*, 1992; Cheung *et al.*, 1994; Saravia-Otten *et al.*, 1997). Analysis of transcription and translation of *fnb* genes demonstrated that synthesis of FnBPA and FnBPB in both an *agr* mutant and wild type took place preferentially during the first hour of growth and rapidly decreased after the second hour. The concentration of *fnb* mRNAs and proteins differed by a factor of 16 between different strains but the difference in fibronectin binding was only twofold, indicating that binding of fibronectin to the bacteria was not directly proportional to the amount of FnBPs on their surface. *fnb* genes were negatively regulated by *agr* and also by an *agr*-independent mechanism that restricted *fnb* mRNA synthesis to the early exponential phase of growth (Saravia-Otten *et al.*, 1997).

The regulation of *fnbA* and *fnbB* by *sar* and *agr* was examined further using single or double mutants of *S. aureus* Newman. Expression of FnBPA was



enhanced in the *agr* mutant but inhibited in the *sar* and the *sar-agr* double mutant. The same regulatory pattern was observed by Northern blot analysis using *fnbA* specific probes. Introduction of *sar* on a multicopy plasmid increased the already enhanced *fnbA* transcription of the *agr* mutant. Thus, *agr* negatively regulates *fnb* gene, while *sar* upregulates *fnb* genes during exponential growth phase. Gel shift assays showed that SarA bound to the *fnbA* promoter fragments. Regulation of FnBPA and FnBPB was not a shared process; FnBPB was not detectable by ligand blotting and the FnBPB promoter activity was not affected by either *sar* or *agr* with only minor amounts of *fnbB*-specific transcripts by Northern blot analysis (Wolz *et al.*, 2000).

An important caveat to such findings is that these experiments represent regulation *in vitro* during growth in broth media. It is becoming increasingly clear that this may not reflect events *in vivo*. Sub-genomic microarray analysis (covering 68 genes) was used to compare gene expression during growth of *S. aureus in vitro* in standard laboratory medium, in rabbit serum *in vitro*, and in chambers implanted into the subcutaneous tissues of rabbits. Expression of RNAIII, the effector molecule of the *agr* locus, was dramatically repressed in serum and *in vivo*, despite the increased expression of secreted virulence factors previously considered to be under negative regulation. A positive correlation between the expression of *agr* and virulence factors was not found, disruption of the *agr* locus resulting in a minimal effect on the expression *in vivo* of those virulence factors examined (Yarwood *et al.*, 2002). Thus, it appears that regulatory circuits *in vivo* override *agr* activity. *sar* promoters have also been shown to be differentially expressed in differing micro-environments. Using green fluorescent protein transcriptional fusions as a detection system for *sar* activation, promoters were found to be differentially expressed *in vitro* and *in vivo*, and to differ depending on the position of the bacteria within an experimental vegetation formed on rabbit heart valve *in vivo* (Cheung *et al.*, 1998).

### 1.3.6 Factors influencing surface expression of functional FnBPs.

There are several possible explanations for the observation that the amount of cell surface FnBP expressed on the bacterial cell surface is maximal during exponential growth but disappears rapidly as the culture progresses into stationary phase. In addition to the process of regulation discussed above, it is also possible that proteins are either masked by a second protein that is secreted during late exponential phase, or that FnBPs are cleaved from the bacterial cell surface after expression has occurred.

#### 1.3.6.1 Masking of FnBPs.

Capsule is well placed to affect adhesion function, especially given the proximity of the D repeats to the bacterial cell wall. Strain M encapsulated (heavily encapsulated) has been shown to bind fibronectin poorly while M unencapsulated bound well. However, Smith diffuse (profuse capsule) and compact (no capsule) had similar binding activity (Switalski *et al.*, 1983). Furthermore, most strains of *S. aureus* produce a microcapsule (at least, under *in vitro* conditions). These observations suggest that capsule may not have a major influence on adhesin function.

Biofilm is another contender as a masking protein. Two genes/gene clusters have so far been implicated in biofilm formation. These are the *icaADBC* operon encoding intercellular adhesion, and *bap* encoding a biofilm-associated protein (Bap), a large protein consisting of 2,276 amino acids (Cucarella *et al.*, 2002). A Bap positive strain was shown to have lower adherence to immobilized fibronectin and fibrinogen than an isogenic deficient strain. The effect of *ica* on adherence to fibronectin is unclear.

Study of FnBPs and ClfA expression by *S. aureus* 8325-4 and Newman genetically altered to carry a chromosomal copy of the *mec* element (encoding methicillin resistance) demonstrated that the presence of *mec* led to defective adherence to fibronectin and fibrinogen. Western blotting showed similar

amounts of protein between mutant and wild type. It was considered possible that the *mec* element also carried a gene encoding a surface protein that masked or interfered with the function of other surface proteins (Vaudaux *et al.*, 1998). This was consistent with previous reports that a higher proportion of methicillin resistant strains failed to agglutinate in the presence of fibrinogen, (a reflection of ClfA function), compared with methicillin <sup>sensitive</sup> strains. Purification of a plasmin sensitive protein from a clinical methicillin resistant *S. aureus* strain (Hilden *et al.*, 1996), was followed by identification of the gene termed *p/s* and characterisation of the cell wall anchored protein which had a serine-aspartate repeat region characteristic of the Clf-Sdr family (Savolainen *et al.*, 2001). A *p/s* mutant constructed by allelic replacement adhered well to immobilized fibronectin (and IgG), in contrast to poor adherence of the parental strain.

#### 1.3.6.2 Proteolytic cleavage of FnBPs.

McGavin *et al.* investigated the cause of poor adherence of one clinical isolate to fibronectin, the results of which suggest that FnBPs are cleaved by serine (V8) protease (McGavin *et al.*, 1997). A culture of a clinical isolate with good fibronectin binding was supplemented at the time of inoculation with concentrated stationary phase supernatant from a second strain with poor binding to fibronectin. The resulting exponential phase cells were devoid of FnBP, but this was prevented by the addition of the universal protease inhibitor  $\alpha_2$  macroglobulin. V8 protease was purified from the supernatant of the poor binder; its addition to a culture of the good binder led to loss of cell surface FnBP and adherence to immobilized fibronectin. V8 protease cleaves on the carboxyl side of glutamic acid (Drapeau *et al.*, 1972), the most abundant amino acid of *S. aureus* FnBP (Signas *et al.*, 1989), and one that is essential for fibronectin binding (McGavin *et al.*, 1991).

The relevance of these observations to *in vivo* events during the course of *S. aureus* infection is not understood. It has been hypothesized that modelling of

the bacterial cell surface may be required during the transition between colonizing and invasive phases of infection with a shift from an adhesive to a relatively non-adhesive phenotype (McGavin *et al.*, 1997), but this is likely to be a gross over-simplification. It is also unclear whether V8 protease-mediated cleavage is common to other strains of *S. aureus*.

### **1.3.7 Interactions between FnBPs and host immunity.**

#### **1.3.7.1 Cellular response.**

Interactions between recombinant FnBP, plasma fibronectin, anti-FnBP antibodies and phagocytic cells (polymorphonuclear cells and macrophages) has been examined (Rozalska & Wadstrom, 1992). Antibodies opsonized and promoted phagocytosis *in vitro*. FnBPs appeared to have chemoattractant activity in the mouse peritoneal cavity, leading to the accumulation of polymorphonuclear cells (PMNs) and macrophages. Phagocytosis of bacteria in the presence of FnBP *in vitro* was the same as that for the control, but the addition of plasma fibronectin led to an increased uptake by macrophages and to a lesser extent PMNs.

Little is known of the effect of FnBPs on other cell types of the immune system, although one study suggests that other interactions may occur; truncated forms of recombinant FnBPA protein have been shown to interact with integrin  $\alpha_5\beta_1$  via a fibronectin bridge to mediate adhesion and co-stimulatory signals to T lymphocytes (Miyamoto *et al.*, 2001).

#### **1.3.7.2 Antibody response.**

One published study has examined the humoral response to FnBPs (Casolini *et al.*, 1998). This was performed using samples from 33 patients with a 'variety' of *S. aureus* infections. Timing of blood samples varied from 2 days - 3 weeks, and the age range of the study group was 21-86 years. Samples from 2 year old children were used as controls. Infected individuals had higher

antibody levels than controls. The antibodies raised preferentially reacted with recombinant D1-4, (regions A, B C and D were tested). Anti-D region antibodies preferentially recognized epitopes in the C-terminal 20 amino acids of the D3 motif, but only once complexed with fibronectin. There was little interaction with recombinant proteins spanning A, B and C regions. All patients had antibodies that specifically recognised the fibronectin-FnBP complex, with epitopes recognized by these ligand induced binding site (LIBS) antibodies in each D repeat of FnBP. Thus, antibodies recognised the antigenic epitope of FnBP after it had undergone a conformational change upon ligand binding. This is consistent with the observation that antibodies did not inhibit fibronectin binding (Casolini *et al.*, 1998).

### **1.3.8 Effect of antibiotics on FnBPs.**

Nearly 20 years ago, Richard Proctor reported that sub-inhibitory concentrations of antibiotics altered fibronectin binding to *S. aureus* (Proctor *et al.*, 1983). This received little further attention at the time but there has been renewed interest in this phenomenon in recent years, principally with respect to fluoroquinolones and the macrolide clindamycin.

Bacterial resistance to fluoroquinolones is mediated through DNA mutation of genes encoding DNA gyrase and topoisomerase IV. A *S. aureus* mutant defective in both *grlA* (topoisomerase IV, A sub-unit) and *gyrA* (DNA gyrase, A subunit) grown in the presence of sub-inhibitory concentrations of ciprofloxacin expressed increased levels of FnBPs and had significantly higher attachment to fibronectin-coated polymer surfaces. This was seen with the double mutant, but not the strains with a single mutation or in the susceptible parent (Bisognano *et al.*, 1997). Fluoroquinolone-resistant clinical isolates were subsequently examined; 8/10 methicillin-resistant and 4/6 methicillin-susceptible strains exhibited a significant increase in attachment to fibronectin-coated surfaces after growth in the presence of one-quarter the MIC of

ciprofloxacin (Bisognano *et al.*, 2000). Enhanced adherence also occurred to explanted coverslips that had been removed from the subcutaneous tissues of guinea pigs, (these become coated with host protein including fibronectin)(Bisognano *et al.*, 2000). Ciprofloxacin increased *fnbB* but not *fnbA* promoter activity of a fluoroquinolone-resistant derivative of 8325 but not its susceptible parent. This was abolished by pre-treatment with rifampicin, indicating an effect at the level of transcription. Activation of the *fnbB* promoter was not due to an indirect effect on growth rate, and still occurred in an *agr* mutant.

Sub-inhibitory clindamycin was reported to eliminate production of nearly all exoproteins but had virtually no effect on cytoplasmic proteins (Herbert *et al.*, 2001). The effect was abolished by a gene conferring resistance to macrolides-lincosamides-streptogramin B, showing that differential inhibition of protein synthesis was responsible. Sub-inhibitory clindamycin stimulated synthesis of FnBPB at the level of transcription, and blocked production of several of the individual exprotein genes, including *spa*, *hla* and *sspA* (encoding protein A, alpha toxin and serine protease, respectively). This pattern is suggestive of differential inhibition of one or more regulatory proteins, but *agr* and *sar* were minimally effected by the antibiotic and did not appear to be responsible.

### **1.3.9 FnBPs and anti-staphylococcal vaccines.**

Much preliminary work has been undertaken towards the development of an anti-staphylococcal vaccine using FnBP as the target. The ideal would be to generate antibodies that block adherence while maintaining the ability to opsonize bacteria and enhance phagocytosis. Whether this is achievable is a topic for debate, reasons for which will become apparent from the discussion below.

Work using experimental animal models started more than a decade ago. D repeats of *S. aureus* FnBPA fused with *E. coli*  $\beta$ -galactosidase (gal-FnBP) was

found to stimulate antibodies in mice which partially blocked the binding of fibronectin to gal-FnBP *in vitro* (Ciborowski *et al.*, 1992). Vaccine efficacy was subsequently examined using several animal models. Rats were immunized with gal-FnBP, after which experimental endocarditis was induced and the outcome compared with unvaccinated controls (Schennings *et al.*, 1993). The bacterial count on the injured heart valve was significantly reduced in the immunized group, and again the antibodies generated partially blocked adherence of *S. aureus* to immobilized fibronectin. Antibodies raised in rabbits to Gal-FnBPA and ZZ-FnBPB and injected into the mouse peritoneum led to a more rapid clearance of *S. aureus* from the peritoneal cavity and liver compared with controls. Clearance of opsonized *S. aureus* from the mouse bloodstream following intravenous injection was also more rapid than that for non-opsonized bacteria (Rozalska & Wadstrom, 1993). Similarly, rabbits immunized with recombinant FnBP had a lower bacterial count in blood and excised organs compared with controls following intravenous inoculation (Park *et al.*, 1999). The protective potential of immunization with fusion proteins encompassing the D repeat region of FnBPA was also evaluated in a mouse mastitis model. Vaccinated mice showed a decreased number of bacteria recovered from mammary glands and significantly reduced cases of severe mastitis (Mamo *et al.*, 1994). These studies suggested that anti-FnBP antibodies were opsonic, but the question remained as to their potential to block the interaction between FnBP and its host ligand.

Antibodies were then analysed in more detail with respect to their ability to block the interaction between FnBP and its ligand, and the epitopes which they recognised. Polyclonal antibodies against recombinant FnBP D repeat (gal-FnBPA and ZZ-FnBPB) were reported to recognize native FnBP on the cell surface (Rozalska *et al.*, 1994). The majority of antibodies in this study recognised a fibronectin-binding D1-D2 sequence of FnBPA, while anti-FnBPA Fab failed to bind the D3 sequence. Anti-FnBPA antibodies blocked fibronectin binding to *S. aureus* by about 50%, the investigators suggested that this might be explained

by the presence of FnBP binding regions outside the D region (Rozalska *et al.*, 1994). In a second study, affinity purified antibodies from rabbits immunized with recombinant D1-D3 were poor inhibitors of fibronectin binding to *S. aureus*; these recognised several different epitopes with a preference for clusters of acidic amino acids that do not contribute to fibronectin binding. A further round of antibodies generated using a GSTD1-3 did not promote more than 50% inhibition. These antibodies were found to be specific for amino acids essential for binding in D1; none recognised the region containing a motif critical for the binding site of D3. Antibodies specific for the binding region of D1 and D3 (raised by immunising with synthetic peptides corresponding to D1<sub>24-34</sub> and D3<sub>20-33</sub> caused the now familiar 40-50% inhibition of binding of *S. aureus* to fibronectin (Sun *et al.*, 1997).

One explanation for the poor ability of antibodies to block the interaction between FnBPs and fibronectin is that antibodies raised *in vivo* are predominantly to ligand-induced binding sites, that is, those that exist only after the FnBP-Fn interaction has occurred. Such antibodies would be expected to have poor or no adherence-blocking activity, and may even enhance ligand binding by stabilizing ligand-FnBP complexes. Furthermore, if anti-LIBS antibodies were present in high concentrations, they could potentially mask inhibiting antibodies, if present. Such thinking has dominated recent work which has aimed to generate antibodies using recombinant FnBP protein with preserved antigenic activity but reduced ligand-binding activity.

D2 peptide of FnBP (amino acids 1-30 of D2 which is unable to bind to fibronectin) was expressed on the surface of the cowpea mosaic virus or potato virus (Rennermalm *et al.*, 2001). High titres of antibodies were obtained from mice and rats immunized with cowpea virus, despite the fact that disordered proteins have been reported to be poor immunogens (Foster & Hook, 1998). Truncated D2 domain of FnBP displayed on a cowpea mosaic virus carrier induced protection against endocarditis in mice, afforded protection from weight



loss due to bacteraemia, and were opsonic *in vitro* (Rennermalm *et al.*, 2001). However, such antibodies were still only capable of partially blocking adherence of *S. aureus* to fibronectin *in vitro* (Brennan *et al.*, 1999).

A similar approach was taken by Huesca and colleagues who set out to eliminate the influence of fibronectin binding on antibody development (Huesca *et al.*, 2000). This was achieved using synthetic peptide immunogens D1<sub>21-34</sub> and D3<sub>20-33</sub> which contain a conserved pattern of amino acids that is essential for fibronectin binding but which cannot bind fibronectin without N- or C-terminal extensions. The D3<sub>20-33</sub> immunogen promoted the production of polyclonal antibodies that were 10-fold more effective as inhibitors of fibronectin binding to the D3 motif than antibodies obtained by immunizing with an extended peptide D3<sub>16-36</sub> which exhibits functional fibronectin binding. The D3<sub>20-33</sub> immunogen also facilitated the production of a monoclonal antibody which was highly specific for the epitope SVDFEED (essential for binding), and which abolished binding by the D3 motif. These investigators concluded that by immunizing with short synthetic peptides that are unable to bind fibronectin, antibodies were raised to epitopes comprised of amino acids that are essential for fibronectin binding. Further work is clearly needed since FnBP contains several ligand binding sites, and blocking antibodies would have to inhibit the binding of ligand to all of these sites if blocking was considered to be an essential property of an antibody response.

## **1.4 Evaluating the role of FnBPs in disease.**

### **1.4.1 Experimental animal models.**

Experimental endocarditis is one of the models commonly used to study *S. aureus* virulence. The aortic valve is damaged by the placement of a catheter via the carotid artery. This stays *in situ* for 24 hours and may be removed or remain in place when a bacterial inoculum is given into a vein. Animals are then sacrificed and the valve examined using bacterial counts. This model has been used to examine the role of FnBPs (Kuypers & Proctor, 1989). Rats inoculated

with a transposon mutant of *S. aureus* 879R4S defective in fibronectin binding were examined after 1 hour by culturing the heart, liver and spleen. Culture of heart valves from catheterised rats had 250 fold fewer organisms after challenge with the mutant, while the number of organisms in other sites was no different. This was repeated using an isogenic mutant of 8325-4 defective in FnBPs (Flock *et al.*, 1996). Adherence of haematogenously spread bacteria to traumatized heart valves at 1 hour was not different between the mutant and wild type. Bacterial counts at 24 hours were comparable, and clearance of the two strains did not differ. It is unclear why these results differ, but a later study using the non-pathogenic *Lactococcus lactis* as a bacterial expression vehicle found that bacteria expressing FnBPA required a lower infective dose to induce valve infection in the rat (Que *et al.*, 2001).

The role of FnBPs in lung infection has been examined using two models. In the first, the trachea of Wistar rats were removed and then frozen and thawed to remove surface epithelium (Mongodin *et al.*, 2002). Primary cultures of human airway epithelial cells were collected from human nasal polyps, cultured *in vitro* and then seeded into the rat tracheas, thereby making a humanized bronchial xenograph. These were implanted into the subcutaneous tissue of the flanks of female nude mice for a variable period before bacteria were inoculated into them, left for one hour, harvested and examined using computer-assisted scanning electron microscopy. Adherence of an FnBP-defective mutant was compared with that of wild type to cells grown to a monolayer *in vitro*, and to the cells lining the implants. Adherence of wild type to non-ciliated and ciliated differentiated epithelial cells was weak *in vitro*, while wild type adhered to undifferentiated cells in a FnBP-dependent manner. This pattern was reproduced using the implants. No bacteria bound to ciliated (differentiated) epithelium, and bacteria were seen to be trapped in mucus. This may have implications for individuals who have damaged epithelium lining the bronchial tree, but it is unclear whether it has relevance to the normal host.

The second lung model used rats inoculated via the respiratory tract, lung injury and bacterial survival being assayed at 24 and 96 hours (McElroy *et al.*, 2002). Growth of *S. aureus* and the extent of lung injury were both increased in rats inoculated with *S. aureus* 8325-4 defective in FnBPs compared with rats inoculated with wild type or the mutant strain re-complemented with *fnb* carried on a multicopy plasmid. FnBP positive bacteria were associated with increased elimination at 24 hours, and reduced damage at 24 hours as defined by protein concentration (as a marker for damage to the air-blood barrier), and RTI<sub>40</sub> (a biochemical marker of type I cell damage). FnBP had no effect on neutrophil recruitment or bacterial uptake by these cells. Thus, FnBP appeared to decrease the virulence of *S. aureus* in pneumonia.

A guinea pig wound abscess model has also been used to study FnBPs, in which the ID<sub>50</sub> did not differ between *S. aureus* 8325-4 and an isogenic mutant defective in FnBPs (Menzies *et al.*, 2002).

#### **1.4.2 *in vitro* models.**

##### **1.4.2.1 Libraries of clinical isolates.**

Previous studies of FnBPs in clinical isolates encompass a relatively limited number of isolates (Proctor *et al.*, 1982; Proctor *et al.*, 1984; Switalski *et al.*, 1983). One study reported adherence of radiolabelled fibronectin to 22 of 23 isolates (Switalski *et al.*, 1983). Isolates from invasive disease have also been reported to agglutinate more readily in fibronectin compared with non-invasive strains, although there were only 11 strains in each group in this study (Proctor *et al.*, 1984). The presence of at least one *fnb* gene has been confirmed for 25 isolates using Southern blot analysis, but whether these possessed one or two *fnb* genes was not determined (Smeltzer *et al.*, 1997). The finding that *S. aureus* 879R4S possesses a single *fnb* gene which is homologous to *fnbA* (Greene *et al.*, 1996), suggests the presence of genetic diversity, but the extent of this is unclear.

#### **1.4.2.2 Interaction with prosthetic material.**

Study of the adherence of *S. aureus* to prosthetic material has principally relied on the use of either segments of intravenous catheter used in an *in vitro* model, or polymethylmethacrylate coverslips implanted into the subcutaneous tissues of guinea pigs and explanted four weeks later. Adherence to explanted coverslips has been reported to be related to fibronectin deposits on the foreign body surface (Vaudaux *et al.*, 1984). *S. aureus* also adhere to unimplanted coverslips coated in fibronectin (Herrmann *et al.*, 1988), or in the presence of serum (Vaudaux *et al.*, 1984). Coating prosthetic catheters *in vitro* with fibronectin or fibrinogen led to increased *S. aureus* adherence (Vaudaux *et al.*, 1993). *S. aureus* also adhered to intravenous catheters collected from adult hospital in-patients. Fibrinogen was found to be present on the surface of these catheters in a degraded form and had poor adherence-promoting properties, while intact or fragmented fibronectin, although in much lower amounts, could actively promote adherence to catheters *in vitro* (Vaudaux *et al.*, 1993). This contrasted with the results of a short term model in which clean catheters were exposed to canine blood for up to 60 minutes. *S. aureus* defective in ClfA (fibrinogen-binding protein) but not FnBPs showed a large decrease in adherence (Vaudaux *et al.*, 1995).

#### **1.4.2.3 Interaction with endothelial cells.**

The mechanism leading to infection of superficial tissues is likely to be through local inoculation. Similarly, the route of entry for organisms causing nosocomial infection occurs primarily via breaches in the normal host defences, for example because of surgery or the presence of an intravenous device. The process by which bacteria gain access to the intravascular compartment to cause community-acquired bacteraemia is unclear, as is the reverse process by which *S. aureus* exit the intravascular compartment to seed deep sites such as bones and joints. This is a common clinical problem; bacterial metastasis from blood to

tissues such as bones, joints and solid organs is clinically apparent in 1-53% of individuals with staphylococcal bacteraemia (reviewed by Ing *et al.*, 1997). It seems likely that bacterial metastasis involves interactions between circulating bacteria and vascular endothelial cells.

*S. aureus* has been shown to adhere to human endothelial cells *in vitro* (Ogawa *et al.*, 1985; Vercellotti *et al.*, 1984). This process was found to be time dependent (Vercellotti *et al.*, 1984), greater for bacteria in exponential versus stationary growth phase (Pohlmann-Dietze *et al.*, 2000; Tompkins *et al.*, 1990; Vriesema *et al.*, 2000), and markedly decreased for bacteria treated with proteases to remove surface proteins (Vercellotti *et al.*, 1984). The heavily encapsulated M strain was reported to adhere poorly, perhaps because of surface masking (Vercellotti *et al.*, 1984), evidence for which was provided by the report that Newman defective in capsule adhered more to endothelial cells than the capsule positive wild type (Pohlmann-Dietze *et al.*, 2000).

Once adhesion has occurred, *S. aureus* cells undergo a process akin to phagocytosis (Lowy *et al.*, 1988; Yao *et al.*, 1995; Vriesema *et al.*, 2000); uptake was blocked by cytochalasin B which interferes with microfilament function (Hamill *et al.*, 1986). Transmission electron microscopy demonstrated that adherence was followed by formation and elongation of surface extensions from the endothelial cell which surrounded and completely enclosed the bacteria during the uptake process (Hamill *et al.*, 1986). Bacterial internalization elicited release of the cytokines IL-6, IL-8 and IL-1 $\beta$  (Yao *et al.*, 1996; Yao *et al.*, 1995). Cytochalasin D, an inhibitor of endocytosis, prevented IL-1 and IL-6 induction (Yao *et al.*, 1995).

Uptake has also been reported to induce surface expression of intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 and monocyte chemotactic protein-1 (MCP-1) (Veltrop *et al.*, 2001; Tekstra *et al.*, 1999), and hyper-adhesiveness for monocytes and granulocytes (Beekhuizen *et al.*, 1997; Veltrop *et al.*, 2001). Endothelial cells expressed tissue factor (TF)-

dependent procoagulant activity (TFA) in response to ingestion of *S. aureus* (Drake & Pang, 1988; Veltrop *et al.*, 1999). Endothelial cells also expressed TFA after exposure to IL-1, but TFA production by *S. aureus* was not prevented by IL-1 receptor antagonist (Veltrop *et al.*, 1999). TF is an obligate co-factor for coagulation factor VII/VIIa and may be important in the development of heart valve vegetations. Supernatants of  $\beta$ -lactam-exposed cultures of *S. aureus* containing cell wall fragments enhanced the adhesiveness of endothelial cells for granulocytes. This coincided with higher ICAM-1 expression, and secretion of IL-8 and MCP-1. These changes may have been due to the action of lipoteichoic acid and peptidoglycan.

Following endothelial cell uptake, *S. aureus* has been demonstrated both within vacuoles and free in the cytoplasm (Menzies & Kourteva, 1998; Ogawa *et al.*, 1985). Intracellular bacteria do not appear to multiply (Vann & Proctor, 1987) although bacterial gene expression studies four hours after internalization have demonstrated activation of genes involved in transport, catabolism, biosynthesis and DNA repair (Vriesema *et al.*, 2000). Internalized bacteria may remain viable for 72 hours (Menzies & Kourteva, 1998). The subsequent fate of the endothelial cell varies between studies. Cells become damaged over time, this depending on the size of bacterial inoculum, length of time after exposure and the strain of *S. aureus* (Vann & Proctor, 1987). Variability in cell damage between bacterial strains may relate to secretion of  $\alpha$ -toxin, a haemolysin which is cidal to endothelial cells (Vann & Proctor, 1988). Lack of  $\alpha$ -toxin production by small colony variants may explain why these can persist in the intracellular niche without causing endothelial cell death (Balwit *et al.*, 1994; von Eiff *et al.*, 1997). Endothelial cell death has been reported to occur by apoptosis, a process dependent on internalization of live bacteria (Menzies & Kourteva, 1998). Whether *S. aureus* can subsequently be released from the basal surface of such an "infected" endothelial monolayer has not been determined. If the endothelial cells are killed before the bacteria, the latter are released and the integrity of the

monolayer is breached. If either mechanism operated *in vivo*, bacteria would gain access to the sub-endothelium.

The majority of clinical isolates produce capsular polysaccharide serotype 5 or 8 (Karakawa & Vann, 1982), which when purified has been shown to bind to endothelial cells and result in release of IL-6 and IL-8 (Soell *et al.*, 1995). However, this is inhibited by the presence of pooled human serum from healthy blood donors (Soell *et al.*, 1995). Endothelial cell activation by IL-1 or endotoxin had no effect on adherence (Blumberg *et al.*, 1988), while human tumour necrosis factor was shown to enhance adhesion to glutaraldehyde-fixed endothelial cells in the presence of plasma (Cheung *et al.*, 1991b).

Attempts to define the endothelial receptor for *S. aureus* adherence have yielded a 50kDa membrane glycoprotein on human cells (Tompkins *et al.*, 1990), and a 130kDa membrane glycoprotein on bovine cardiac endothelial cells (Johnson, 1993). Neither has been characterized further. Fibrinogen had been reported to act as a bridging molecule in adherence to glutaraldehyde fixed endothelial cells (Cheung *et al.*, 1991a), without definition of the cognate ligands for it on either cell type. The bacterial determinants that promote adhesion of *S. aureus* to endothelium had not been elucidated prior to the start of this thesis.

## **1.5 Fibronectin-binding proteins and *S. schleiferi*.**

### **1.5.1 Clinical manifestations of *S. schleiferi* infection.**

Following the description of *Staphylococcus schleiferi* subsp. *schleiferi* in 1988 (Freney *et al.*, 1988), this coagulase-negative staphylococcus has been identified from in-patient microbiological specimens, skin flora, and the hospital environment. A proportion of positive cultures arising from patient specimens appear to have no clinical significance (Fleurette *et al.*, 1989; Vandenesch *et al.*, 1994). However, *S. schleiferi* has been clearly implicated as the causative pathogen in a range of nosocomial infections including bacteraemia (Jean-Pierre *et al.*, 1989; Latorre *et al.*, 1993), brain abscess (Grattard *et al.*, 1993), pacemaker and other intravenous device-related infections (Celard *et al.*, 1997; Da Costa *et al.*, 1998; Grattard *et al.*, 1993), orthopaedic implant-associated infection (Jean-Pierre *et al.*, 1989), wound infection (Grattard *et al.*, 1993), and urinary tract infections (Ozturkeri *et al.*, 1994). This organism has also been associated with an outbreak of surgical wound infections in 6 patients undergoing cardiac surgery (Kluytmans *et al.*, 1998).

### **1.5.2 Pathogenic mechanisms associated with *S. schleiferi* infection.**

The processes leading to *S. schleiferi* infection are unknown, but given the degree of similarity between the spectrum of infections caused by this microorganism, and those associated with *S. aureus* (Waldvogel, 2000), it is possible that the two species express one or more common virulence determinants. *S. aureus* fibronectin- and fibrinogen binding proteins may play a central role in the pathogenesis of colonization of medical devices through the interaction with fibrinogen and fibronectin that rapidly coats prosthetic material following insertion *in vivo* (Vaudaux *et al.*, 1989; Vaudaux *et al.*, 1993; Vaudaux *et al.*, 1995). The clinical observation that *S. schleiferi* has been isolated from cultures of prosthetic material (Celard *et al.*, 1997; Grattard *et al.*, 1993; Jean-Pierre *et al.*, 1989; Vandenesch *et al.*, 1994), suggests the presence of one or



more bacterial cell surface-expressed adhesins with a similar host protein specificity. *S. schleiferi* has been reported to bind fibrinogen as assessed by commercial agglutination kits (Hebert, 1990; Personne *et al.*, 1997; Vandenesch *et al.*, 1994), but adherence to fibronectin and the identification of cell wall-associated adhesins have not previously been described for this organism.

~~thesis~~.

## 1.6 Aims of this dissertation.

~~thesis~~

The work contained in this ~~dissertation~~ describes a series of studies that explore the role of fibronectin-binding proteins (FnBPs) in human staphylococcal disease. Specifically, these projects:

1. Examine the role of *S. aureus* FnBP in adherence to, and invasion of human endothelial cells *in vitro*.
2. Examine the role of each domain of FnBPA in the interaction with endothelial cells.
3. Determine whether endothelial cell expressed integrins are involved in *S. aureus* uptake.
4. Examine whether proteases, and in particular serine (V8) protease, influence FnBP function, potentially modelling the bacterial cell surface and controlling the presence or absence of a functional adhesin.
5. Demonstrate the presence of a functional FnBP by the nosocomial pathogen *Staphylococcus schleiferi*.
6. Study phenotypic and genotypic variation in *fnb* genes in a large population of clinical isolates.
7. Determine the presence of *fnbA* encoding *S. aureus* FnBPA, together with 32 other bacterial factors, in a second bacterial population associated carriage and invasive disease. This was undertaken to identify virulence-associated genes (one of which was *fnbA*); to assess the cumulative effect of virulence-associated genes on virulence; to identify gene combinations, and determine if some combinations have a greater pathogenic potential than others; and to study the horizontal transfer of putative virulence determinants.

## **Chapter 2. Materials and Methods.**

### **2.1 Chemicals and reagents.**

All chemicals and reagents were obtained from the Sigma-Aldrich Company Ltd., unless otherwise indicated. Culture media (Medium M199, phosphate-buffered saline and fetal calf serum) were from Gibco Life technologies Ltd. Falcon tissue culture plasticware was obtained from Becton Dickinson Ltd.

### **2.2 Bacterial culture and storage conditions.**

(a) *Liquid media*: *S. aureus* and coagulase-negative staphylococci were grown in Todd Hewitt broth (THB) or Trypticase soy broth (TSB) under constant rotation at 37°C in air. *Escherichia coli* was cultured in Luria-Bertani (LB) broth under constant rotation at 37°C in air. *L. lactis* MG1363 was grown in M17 broth containing glucose (0.5%, w/v) static at 30°C in air. Cultures were routinely grown in 10ml of media contained in 35ml glass universal containers. Bacteria cultured prior to harvest of cell wall-associated proteins were grown in 25ml brain heart infusion (BHI) in 100ml flasks.

(b) *Solid media*: *S. aureus* and coagulase-negative staphylococci were grown on either trypticase soy agar (TSA) or 5% horse blood agar. *E. coli* was grown on Luria agar, and *L. lactis* on M17 agar. Agar plates were incubated at 37°C in air. Antibiotics were incorporated into media, where appropriate, at the following concentrations: chloramphenicol 10µg/ml; ampicillin/50µg; tetracycline 2µg/ml; and erythromycin 5µg/ml. Phage base plates and phage agar were used for bacterial transduction.

(c) *Bacterial storage*: *L. lactis* was suspended in M17 broth containing glucose (0.5%, w/v) and glycerol (15%, v/v). *S. aureus* and coagulase-negative

staphylococci were suspended in TSB containing glycerol (15%, v/v), and *E. coli* in LB broth containing glycerol (15%, v/v). All isolates were stored at  $-80^{\circ}\text{C}$ .

Media was from obtained from Difco Laboratories and prepared by the Department of Microbiology, John Radcliffe Hospital who undertook weekly quality control.

## **2.3 Methodology used in studies of bacterial adherence to host proteins.**

### **2.3.1 Adherence of staphylococci to purified human fibronectin.**

Previous investigators have studied adherence of staphylococci to plasma and matrix proteins *in vitro* using one of two methods: (i) adhesion to protein in solution, adherence being detected by the use of radiolabelled bacteria; or (ii) adherence of bacteria to host proteins absorbed onto a plastic surface, adhesion being measured either visually or by optical density after bacterial staining with Crystal violet. These methods are not directly comparable since adherence of a given isolate may be inconsistent between the two types of assay, (for example, the laboratory isolate *S. aureus* Newman adheres to soluble fibronectin but adhesion to immobilised fibronectin is poor) (Vaudaux *et al.*, 1995). There is no consensus as to which assay should be adopted for the study of staphylococci. It was decided to use adherence to immobilised proteins throughout this study, thereby avoiding the used of radioisotopes and providing a permanent record of the result.

### **2.3.2 Growth of bacteria for use in adherence assays.**

Staphylococci normally form microscopic clumps during growth *in vitro*. This makes visual counting of bacterial adherence to proteins or host cells difficult. It could also give misleadingly high results for the number of adherent bacteria as assessed by either visual or indirect enumeration methods, since a proportion may simply be part of a clump. Could the clump size be reduced by gentle washing of a bacterial suspension? To answer this, the laboratory strain *S.*

*aureus* 8325-4 was inoculated from frozen stocks into 10ml THB and incubated for 18 hours under constant rotation at 37°C in air. Following centrifugation at 3,000 rpm for 10 minutes, the bacterial pellet was placed into a 1.5ml eppendorf and washed four times in phosphate-buffered saline (PBS) by gently pipetting up and down using a Gilson pipette. Bacteria from the unwashed overnight culture and each of the washing steps were Gram stained. Bacteria from the overnight culture were clumped as expected. Each wash reduced the average size of the clump until bacteria washed four times were in singles, pairs or small clumps. All bacterial preparations used in adherence assays (to both host proteins and endothelial cells), subsequently underwent four washes in PBS prior to use.

### **2.3.3 Solid-phase fibronectin bacterial adherence assay.**

The first objective was to standardise the adherence assay in such a way that made it robust and reproducible, and able to detect differences in adherence between bacterial strains. The following variable parameters were considered: (1) duration and conditions of bacterial culture prior to adherence assay; (2) incubation conditions and duration of adherence assay; (3) size of bacterial inoculum used in adherence assay; and (4) concentration of fibronectin. Parameters (1) and (2) were set at this point, growth of bacteria used in the assay being standardised to 15-18 hours shaking at 37°C in air, and incubation conditions for the adherence assay itself fixed at 1 hour static at 37°C in air. Parameters (3) and (4) were evaluated in pilot studies in which adherence to fibronectin was examined using variable inocula and a range of protein concentrations, as follows.

(a) *Standardising the inoculum:* *S. aureus* 8325-4 was grown in 10ml THB for 18 hours under constant rotation at 37°C in air, collected by centrifugation, washed four times in PBS and resuspended to give a series of suspensions with a range of spectrophotometer readings at OD<sub>650</sub>. Aliquots of 100µl from each sample

were spread over a blood agar plate (in triplicate), and incubated overnight at 37°C in air. The number of colonies were counted and the weighted mean count calculated (British Standards Institution, 1981). Figure 2.1 shows the results for colony forming units per ml (cfu/ml) versus OD<sub>650</sub> of bacterial suspension. This provided the basis for resuspending washed staphylococci to a known concentration.

Human fibronectin 10µg/ml in PBS was spotted onto tissue culture grade 60 x 15mm plastic petri dishes. These were incubated at 37°C in air for 1 hour, then flooded with bovine serum albumin (BSA, 1%, v/v) in PBS and maintained overnight at 4°C. The dishes were rinsed twice in PBS immediately prior to use. Bacterial suspensions of overnight, washed cultures of *S. aureus* 8325-4 were adjusted by optical density (OD<sub>650</sub>) followed where necessary by serial dilution to a final concentration of  $1 \times 10^6$ ,  $1 \times 10^7$ ,  $5 \times 10^7$  or  $1 \times 10^8$  cfu/ml in PBS with BSA (1%, v/v). After adding 1.5ml of a given suspension, dishes were incubated at 37°C in air for 1 hour, rinsed four times with PBS, fixed with glutaraldehyde (2%, v/v) in PBS for 2 hours and stained with Crystal violet (0.5%, v/v) for 5 minutes. Adhesion was then quantitated using a visual method. The purified fibronectin spots were examined using a calibrated graticule under oil immersion with a X100 magnification lens. The number of bacteria adherent to an area of 1mm<sup>2</sup> of purified protein was enumerated by a standardised counting procedure. Without prior visual inspection, 5 high power fields were selected for counting as follows: (1) the centre of the protein spot; (2) two points between the centre and left edge; and (3) two points between the centre and right edge. Each spot was performed in quadruplicate in a given experiment, and independent experiments were performed three times, the results for which are shown in Figure 2.2.

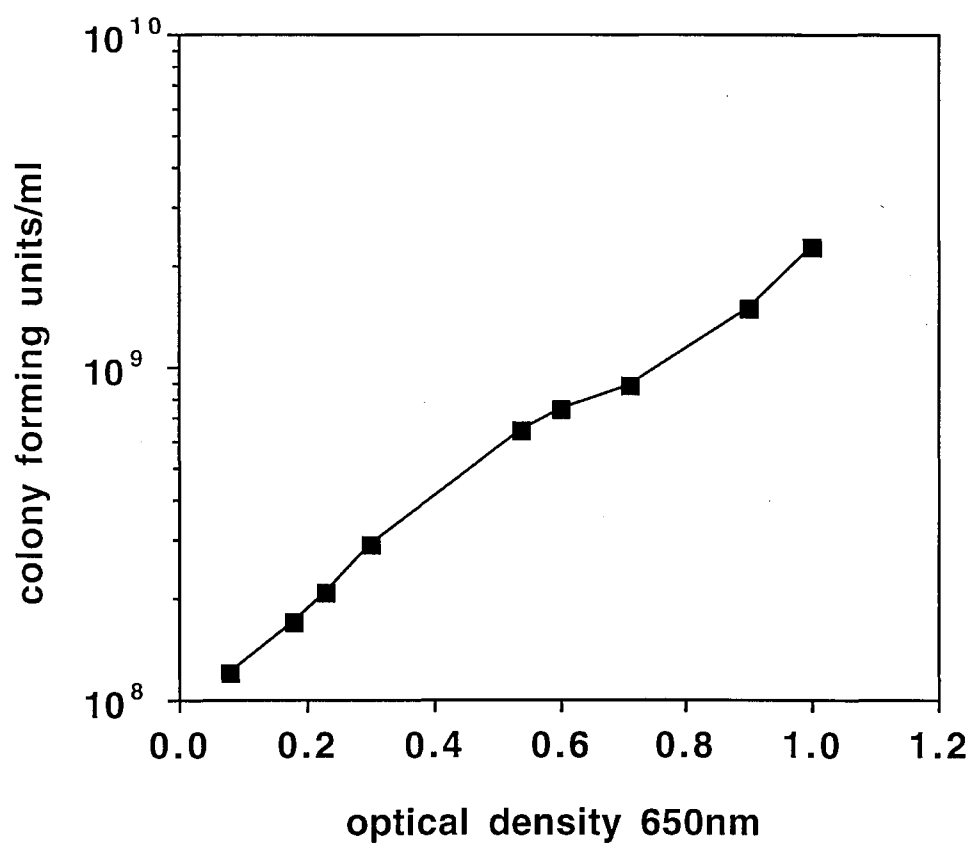
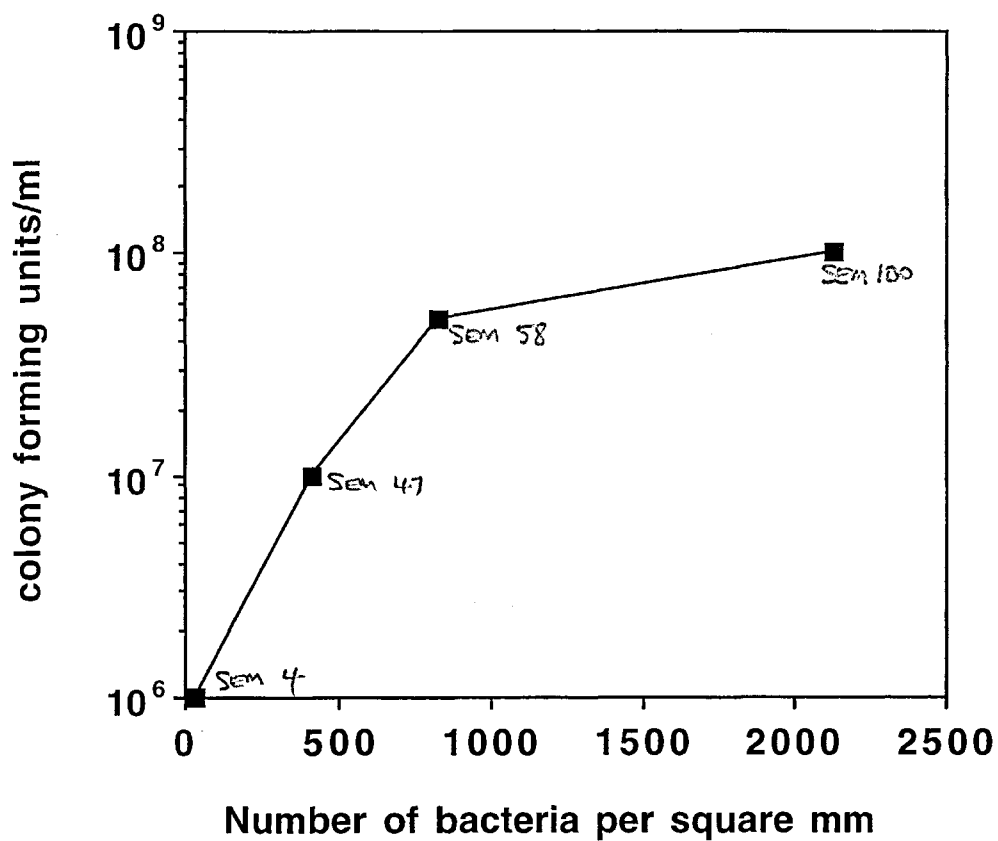


Figure 2.1 Colony forming units/ml of *S. aureus* 8325-4 in PBS versus OD<sub>650</sub> of bacterial suspension.

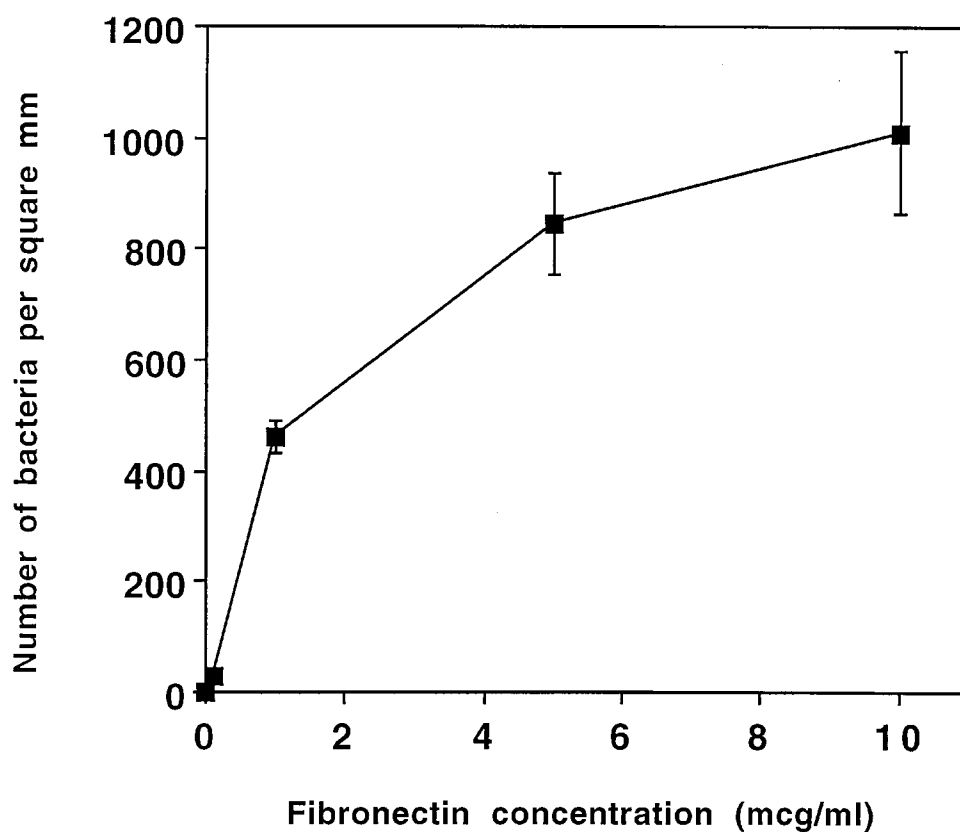


**Figure 2.2 Bacterial inoculum versus number of bacteria (*S. aureus* 8325-4) adherent to  $1\text{mm}^2$  of petri dish base coated with purified fibronectin  $10\mu\text{g/ml}$ . Results represent the mean of triplicate experiments.**



(b) *Standardising the concentration of fibronectin:* Human fibronectin 1µg/ml, 5µg/ml or 10µg/ml in PBS was spotted onto tissue culture grade 60 x 15mm plastic petri dishes. Plates were incubated and rinsed as described in (a) above. A bacterial suspension of an overnight, washed culture of *S. aureus* 8325-4 at  $5 \times 10^7$  cfu/ml in PBS with BSA (1%, v/v) was added to the dish, incubated, rinsed, fixed and counted as described in (a) above. Each spot was performed in quadruplicate in a given experiment, and independent experiments were performed three times, the results for which are shown in Figure 2.3.

Subsequent use of this type of adherence assay used a bacterial suspension of  $5 \times 10^7$  cfu/ml and fibronectin 10µg/ml. The reasons for this were that: (i) this gave countable numbers of bacteria in the range of approximately 1000 bacteria per  $1\text{mm}^2$  of protein for wild type bacteria; and (ii) results lay on the steep part of the dose response curve. Differences in adhesion (either up or down) between the control and other bacterial strains should, therefore, be detectable.



**Figure 2.3** Number of bacteria (*S. aureus* 8325-4) adherent to 1mm<sup>2</sup> of petri dish base versus fibronectin concentration (bacterial concentration 5 x 10<sup>7</sup> cfu/ml). Results represent mean ± standard error of the mean (SEM) of triplicate experiments.

#### **2.3.4 Microtitre plate fibronectin adherence assay using a non-visual method to determine bacterial adhesion.**

The adherence assay above was applicable to small numbers of bacterial isolates (and is used for work in chapter 3), but was not time efficient when testing adherence of large numbers of bacterial isolates to fibronectin. A method was subsequently used for the remainder of the work based on adherence to protein coating the base of 96 well microtitre plates, in which adherence was assessed by optical density after staining of bacteria with Crystal violet.

Pilot studies were performed to determine working concentrations of bacteria and fibronectin. Growth of bacteria used in the assay was standardised from the outset to 15-18 hours shaking at 37°C in air, and incubation conditions for the adherence assay itself were fixed at 1 hour static at 37°C in air. Ninety-six well flat-bottomed polystyrene microtitre plates (Corning Costar, NY) were coated for 1 hour at 37°C with 100µl of purified fibronectin at a concentration ranging from 0.001µg/ml to 100µg/ml. Remaining sites were blocked by overnight incubation at 4°C with 200µl BSA (1%, v/v) in PBS. The plates were washed three times with PBS prior to inoculation of 100µl washed bacterial suspension at a concentration of 10<sup>6</sup>, 10<sup>7</sup>, 10<sup>8</sup> or 10<sup>9</sup> cfu/ml. After incubation at 37°C in air for 1 hour, the wells were rinsed three times with PBS and bound cells were fixed with 200µl glutaraldehyde (2%, v/v in PBS) for 60 minutes and stained with Crystal violet (0.5%, v/v) for 5 minutes. The plates were rinsed with water, air-dried, and the absorbance measured at OD<sub>405</sub> using an ELISA plate reader. Each fibronectin/bacterial concentration combination was performed in quadruplicate in a given experiment, and independent experiments were performed three times. All assay plates included PBS without bacteria as a negative control. The OD<sub>405</sub> was taken as the mean value for a given isolate after subtraction of the background, (defined by the OD<sub>405</sub> of the negative control on the same plate). The results are shown in Figure 2.4. Thereafter, this assay used 100µl of bacterial suspension at 1 x 10<sup>9</sup> cfu/ml and fibronectin 10µg/ml.

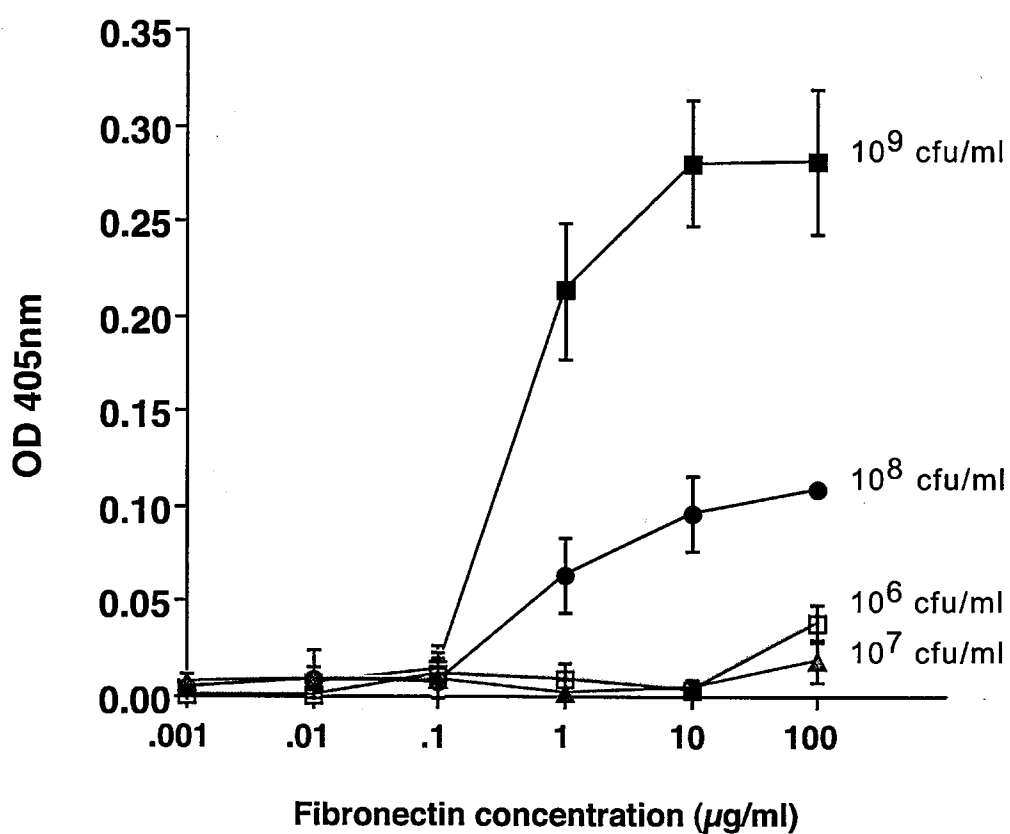
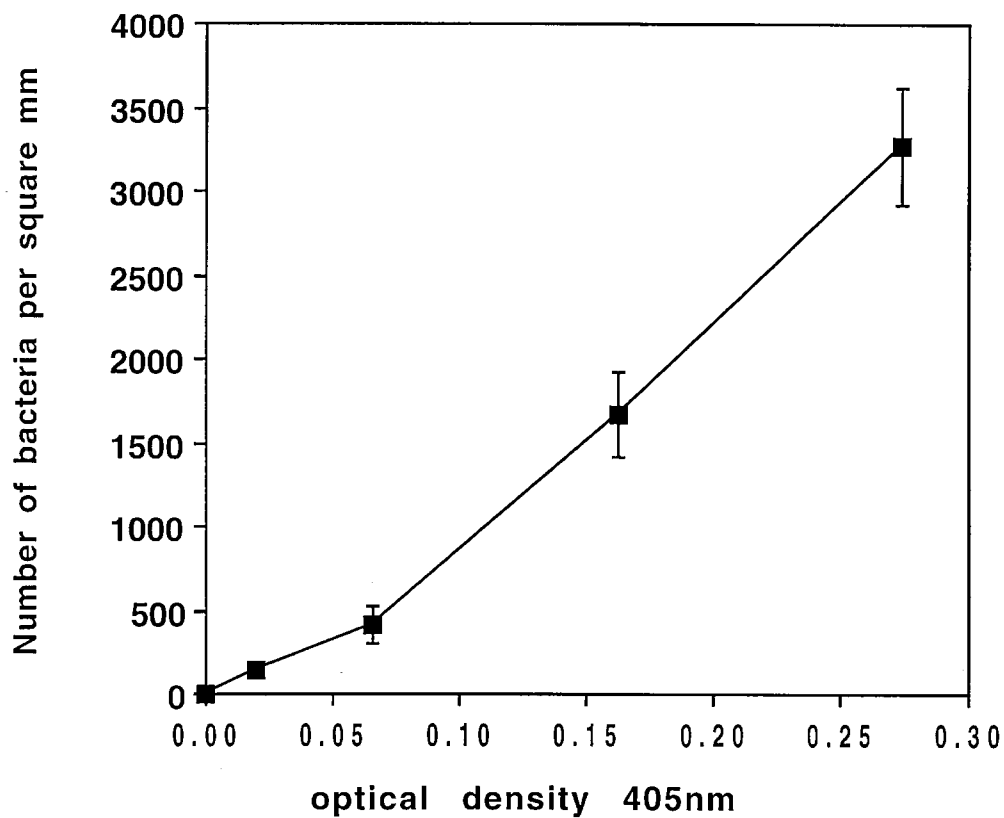


Figure 2.4 Microtitre plate adherence assay: optical density (OD<sub>450</sub>) versus bacterial inocula (*S. aureus* 8325-4) and fibronectin concentration. Results represent mean  $\pm$  SEM of triplicate experiments.

### **2.3.5 Relationship between OD<sub>405</sub> in the microtitre assay and the number of adherent bacteria.**

Making sense of different optical density results between bacterial isolates examined using a non-visual microtitre plate adherence assay depends on an understanding of the relationship between OD<sub>405</sub> and the actual number of adherent bacteria. This was evaluated as follows. Adhesion of *S. aureus* 8325-4 to fibronectin 10µg/ml was performed for a range of bacterial inocula ( $10^5$ ,  $10^6$ ,  $10^7$ ,  $5 \times 10^7$  and  $10^8$  cfu per well in 100µl volumes) using a modification of the assay described above. Fibronectin was adsorbed onto 13mm Thermanox coverslips (Gibco) which were trimmed to fit the bottom of 96-well assay plates. After the OD<sub>405</sub> were read the coverslips were mounted onto glass slides, and the number of bacteria adherent to a surface area of 1mm<sup>2</sup> counted using a calibrated graticule under oil immersion at X100 magnification. Quadruplicate coverslips were used per assay at each bacterial concentration, and independent assays were performed in triplicate. The results obtained demonstrated a linear relationship between OD<sub>405</sub> and adherence to fibronectin over the range of OD<sub>405</sub> values obtained for clinical isolates (Fig. 2.5).



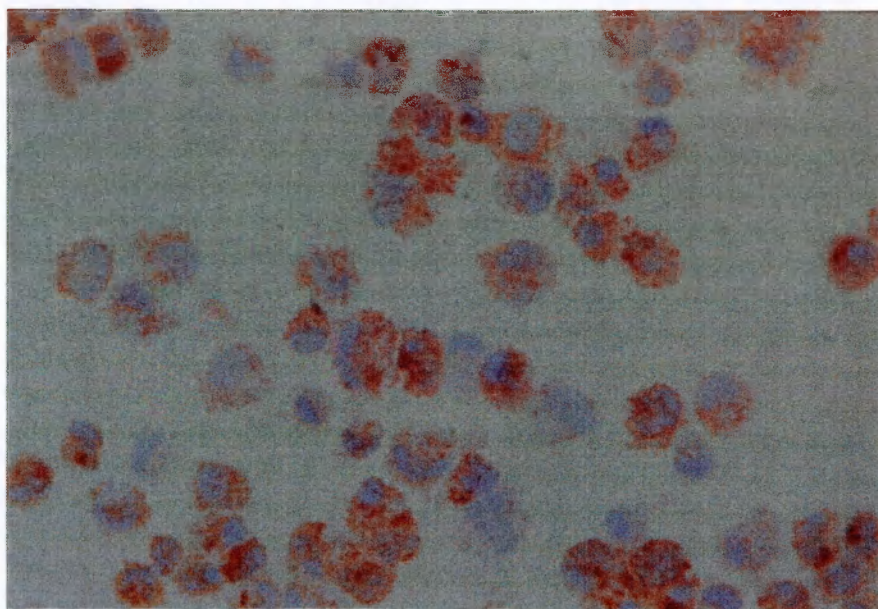
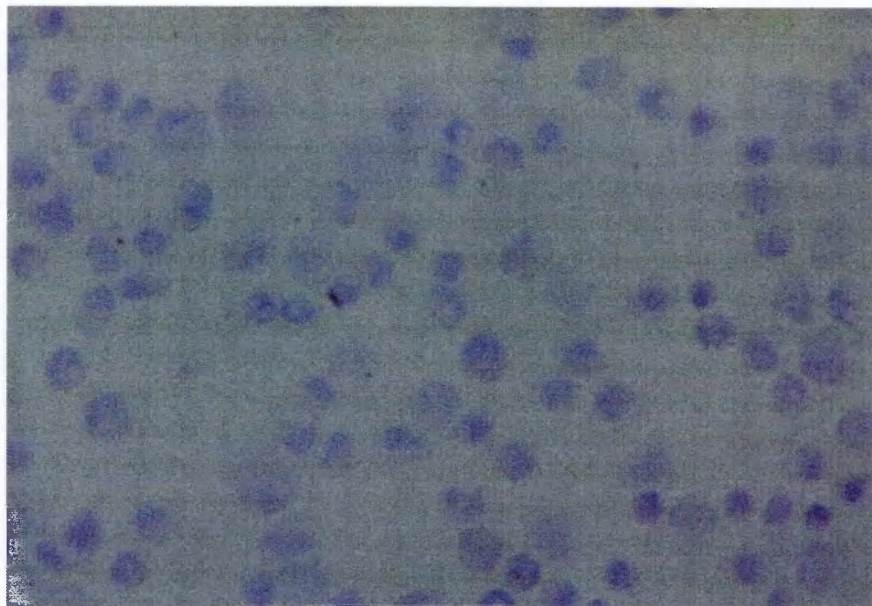
**Figure 2.5** Number of bacteria (*S. aureus* 8325-4) adherent to 1mm<sup>2</sup> of Thermanox coverslip coated with purified fibronectin 10μg/ml versus optical density (OD<sub>405</sub>). Results represent mean ± SEM of triplicate experiments.

## **2.4 Endothelial cell assays.**

### **2.4.1 Endothelial cell culture.**

Endothelial cells were obtained from human newborn umbilical vein using an adaptation of a previously described method (Jaffe & Mosher, 1978). Both ends of the cord vein were cannulated using a Portex luerlock adaptor (Southern Syringe Ltd.), after which the vessel was flushed with PBS. Cells were released by instilling M199 supplemented with penicillin 50U/ml (Gibco), streptomycin 50µg/ml (Gibco) and collagenase type IA 0.5mg/ml into the vessel lumen. After incubation for 20 minutes at 37°C in 5% CO<sub>2</sub> the cell suspension was collected by centrifugation, resuspended in M199 supplemented with fetal calf serum (20%, v/v), heparin 90µg/ml, recombinant fibroblast growth factor 5ng/ml, penicillin 50U/ml and streptomycin 50U/ml and seeded into a 25cm<sup>2</sup> tissue culture flask. Cells were maintained at 37°C in 5% CO<sub>2</sub> and passaged twice on reaching confluence using a 1:3 split, before subculture onto 13mm Thermanox coverslips in 24-well tissue culture plates. All flasks and coverslips used in cell culture were pre-coated with gelatin (0.2%, v/v) overnight at 37°C.

The identity of the cells was confirmed as endothelial by their cobblestone appearance at confluency, and positive staining for von Willebrand factor (vWF) (Jaffe *et al.*, 1973). In brief, confluent endothelial cells were lifted from the tissue culture flask by incubating with 2mM EDTA in PBS, resuspended in growth media and spun onto a glass slide using a cytospin. After drying for 8 hours at room temperature, cells were fixed with acetone and incubated with mouse antibody to vWF (1:250, Dako) for 30 minutes. After rinsing, horseradish peroxidase-conjugated goat anti-mouse antibody was added (1:200, Dako) for 30 minutes followed by DAB (peroxide substrate, 3-3 diaminobenzidine). A negative control (no mouse antibody to vWF) was performed in parallel. Slides were dipped in haematoxylin to give background staining of the cells, then covered with coverslips using aquamount. Representative views of the results are shown in Figure 2.6.



**Figure 2.6 Staining of human endothelial cells for von Willebrand factor.** Top view, negative control; bottom view, cytoplasmic staining of von Willebrand factor. Light microscopy, X40 magnification.



#### **2.4.2 Immunofluorescence of endothelial cell surface receptors.**

A brief description of the technique will be given here, as a more detailed account of the receptors examined has been provided in chapter 3. Confluent endothelial cells on Thermanox coverslips were dip-washed three times and placed into 24-well plates containing 500µl M199. Added to this was a mouse monoclonal or rabbit polyclonal antibody recognizing the receptor of interest at a dilution of 1:200. Coverslips were incubated at 37°C in CO<sub>2</sub> for 30 minutes, dip-washed, and placed into fresh 24-well plates containing FITC-labelled anti-mouse antibody (F0479, Dako) or anti-rabbit antibody (API32F, Chemicon) 1:200 in M199. After a further 30 minutes at 37°C in CO<sub>2</sub>, cells were rinsed, the coverslips mounted onto glass slides and examined using a fluorescence microscope (Zeiss).

#### **2.4.3 Bacterial preparation for endothelial cell adherence assay.**

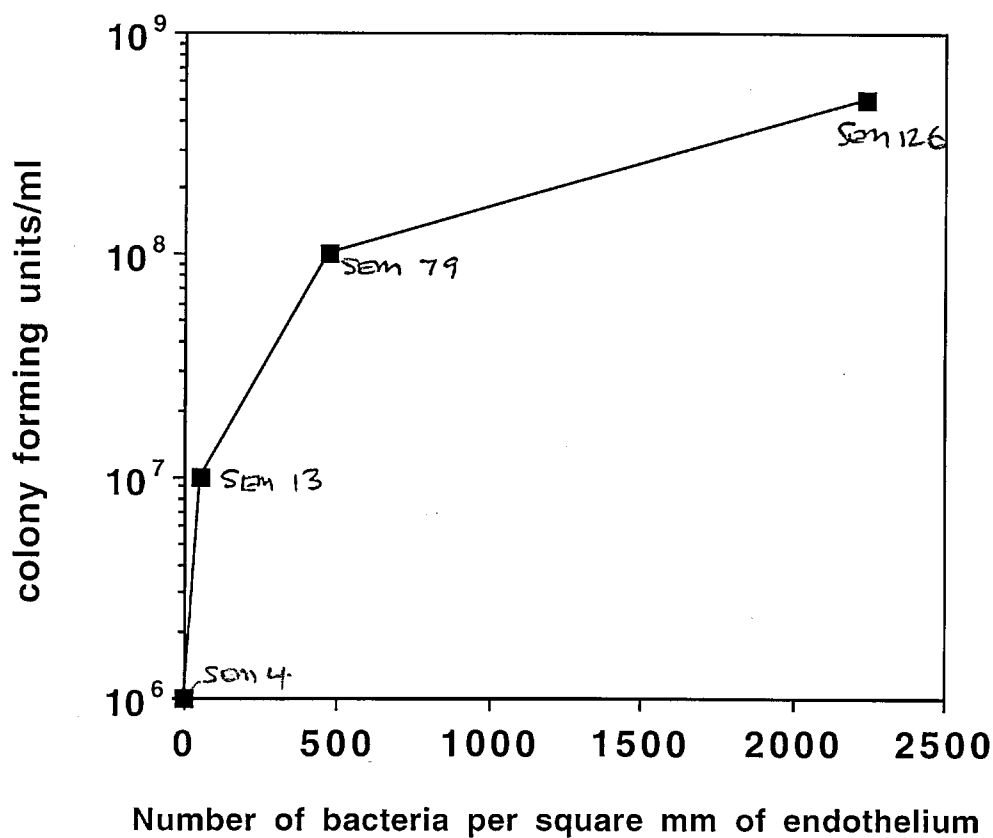
*S. aureus* isolates were inoculated from frozen stocks into THB and incubated for 15 to 18 hours under constant rotation at 37°C in air. *S. aureus* containing plasmids were cultured in THB supplemented with antibiotics to maintain plasmid selection, as appropriate. Bacteria were collected from broth culture by centrifugation at 3,000 rpm for 10 minutes, washed four times with PBS, resuspended in Medium M199 with Earle's salt and 25mM HEPES, (M199) and filtered through a sterile 5µm filter (Gelman Sciences) to remove any remaining bacterial aggregates. A subsequent Gram stain showed the cell suspension to comprise bacteria singly or in pairs. Final bacterial concentrations were adjusted to an OD<sub>650</sub> of 0.90-0.94 corresponding to 1 x 10<sup>9</sup> cfu/ml.

#### **2.4.4 Endothelial cell adherence assay.**

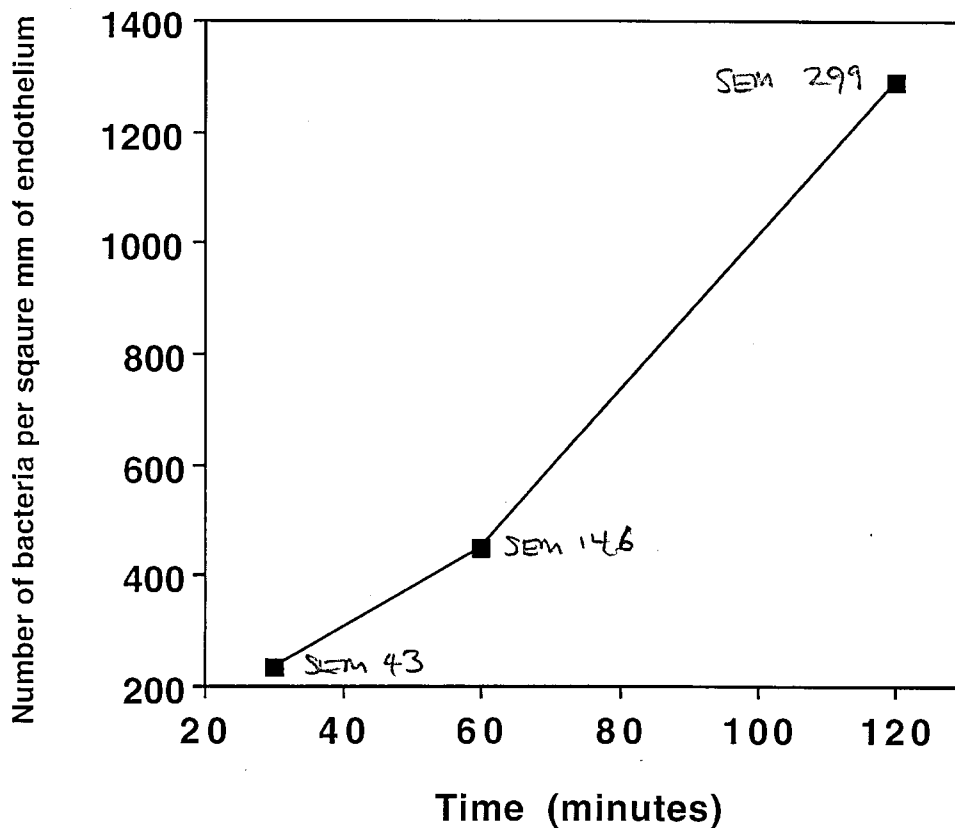
Sterile 24-well flat-bottomed tissue culture plates were blocked with BSA (1%, v/v) for 1 hour and rinsed twice with PBS. A bacterial inoculum of 10<sup>8</sup> cfu suspended in 500 µl M199 was added to each well. This inoculum was selected on the basis of dose response curves, the results for which are shown in Figure 2.7. Confluent endothelial cells coating 13mm Thermanox coverslips were added after dip-washing three times in M199 to remove traces of culture media. The 24-well plates were incubated at 37°C in CO<sub>2</sub> for 1 hour. This incubation period was selected on the basis of time course studies, the results for which are shown in Figure 2.8. The coverslips were then dip-washed three times in M199 and once in PBS to remove non-associated bacteria. The endothelial cells were fixed with Cytofix, air dried, and stained with Crystal violet (0.5%, v/v) for five minutes. Following dip rinsing in water the coverslips were air dried and mounted on glass slides.

The number of endothelial cell-associated bacteria were quantitated using a visual method. The bacterial count included both adherent and internalized bacteria, but for simplicity bacteria are referred to as adherent in the remainder

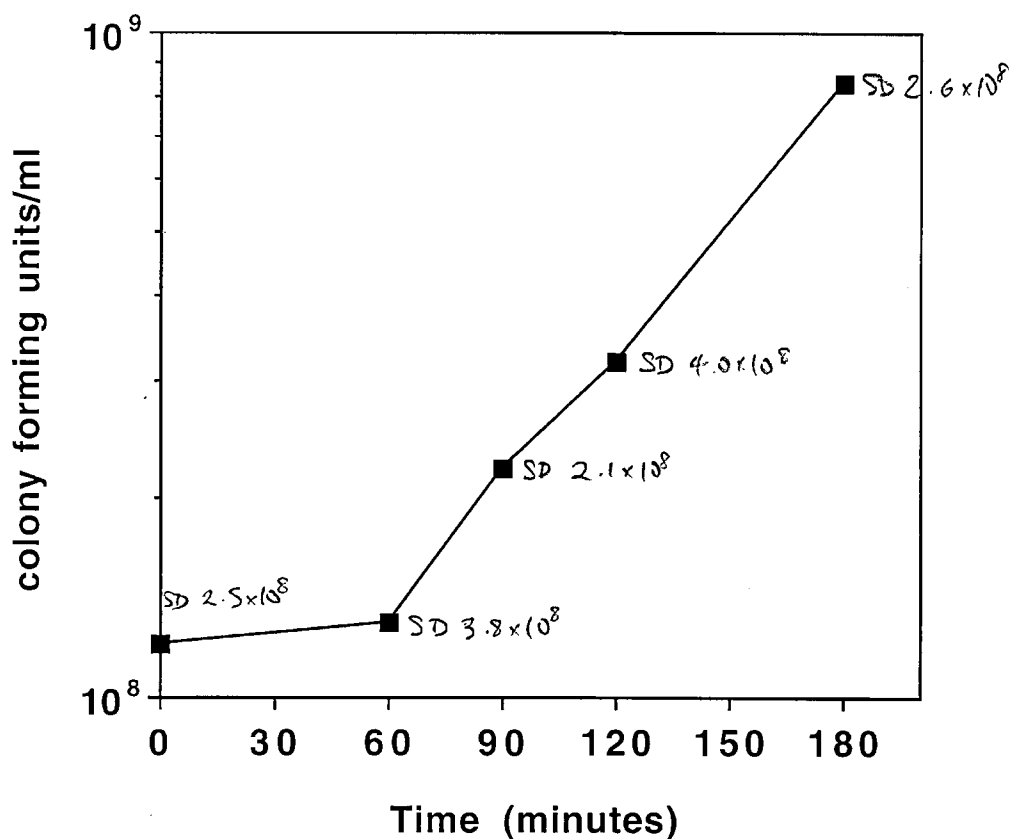
of the text when referring to this assay. Bacteria prepared for the adhesion assay as described above were shown not to divide over the course of an hour in binding medium (see Fig. 2.9), making the number of visualized bacteria an accurate representation of those that had adhered. Each coverslip was scanned under low power to ensure confluence and integrity of the monolayer. Using a calibrated graticule under oil immersion and a X100 magnification lens, the number of bacteria associated with  $1\text{mm}^2$  of confluent endothelial cells was enumerated by a standardised counting procedure. Without prior visual inspection at high power, 5 fields were selected for counting as follows: (1) the centre of the coverslip; (2) two points between the centre and left edge; and (3) two points between the centre and right edge. Light microscopy views of the endothelial cell monolayer following a standardised adherence assay are shown in Figure 2.10.



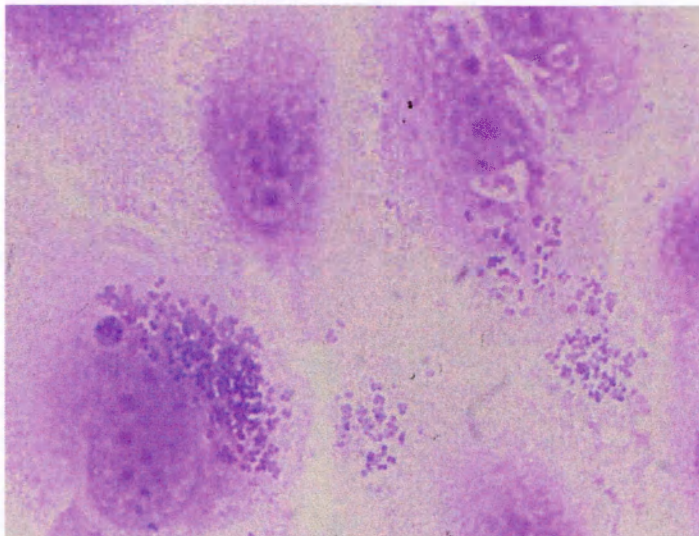
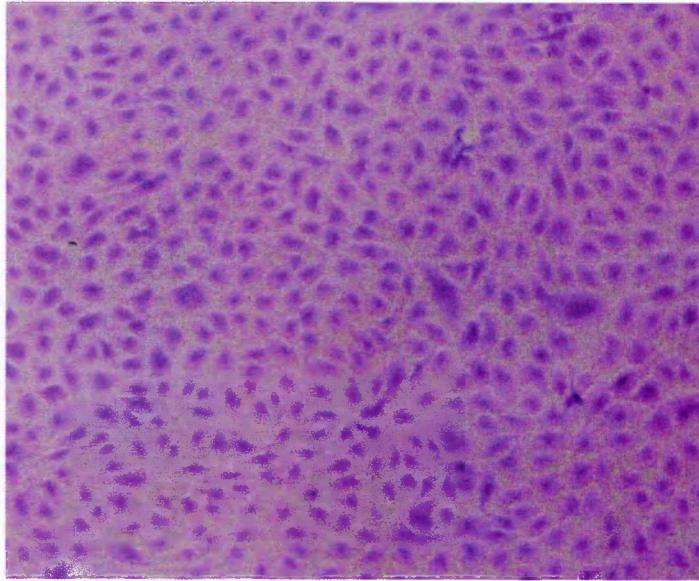
**Figure 2.7** Number of bacteria (*S. aureus* 8325-4) adherent to  $1\text{mm}^2$  of endothelial monolayer over a range of bacterial inocula. Bacterial inocula of  $10^6$ ,  $10^7$ ,  $10^8$ , or  $10^9$  cfu per well were added to confluent endothelial cells coating 13mm Thermanox coverslips in 24-well plates. Coverslips were washed, fixed and stained after incubation at  $37^\circ\text{C}$  in  $\text{CO}_2$  for 1 hour. The bacterial count per  $\text{mm}^2$  of endothelium was enumerated visually. Results represent the mean of triplicate experiments.



**Figure 2.8** Number of bacteria (*S. aureus* 8325-4) adherent to 1mm<sup>2</sup> of endothelial monolayer over an incubation time course. A bacterial inoculum of 10<sup>8</sup> cfu per well was added to confluent endothelial cells coating 13mm Thermanox coverslips in 24-well plates. Coverslips were washed, fixed and stained after incubation at 37°C in CO<sub>2</sub> for 30, 60 or 120 minutes. The bacterial count per mm<sup>2</sup> of endothelium was enumerated visually. Results represent the mean of triplicate experiments.



**Figure 2.9 Growth curve of *S. aureus* 8325-4 following suspension in binding media used in endothelial cell adherence assay.** A washed bacterial suspension at a concentration of  $10^8$  cfu/ml in binding media was incubated at  $37^\circ\text{C}$  in  $\text{CO}_2$  (to reproduce growth conditions used in the endothelial cell adherence assay). The subsequent growth curve was examined over a time course of 180 minutes using agar plate counts. Results represent the mean of triplicate experiments.

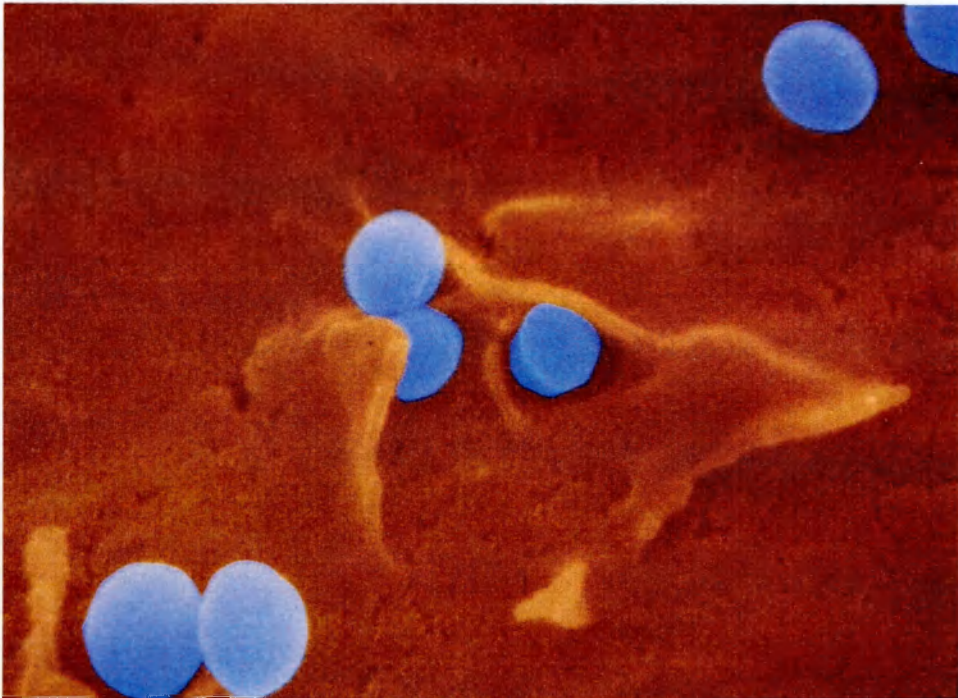
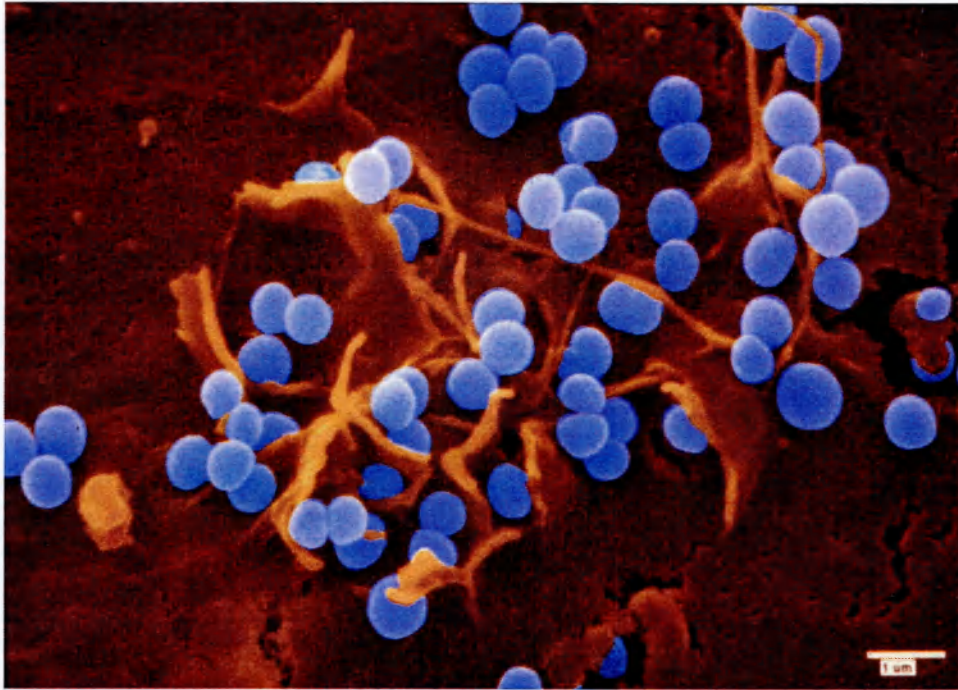


**Figure 2.10 Endothelial cell monolayer under light microscopy.** Top view, endothelial cells sham-treated (no bacteria) during the assay, X10 magnification; bottom view, endothelial cells following standardised adherence assay, X100 magnification.

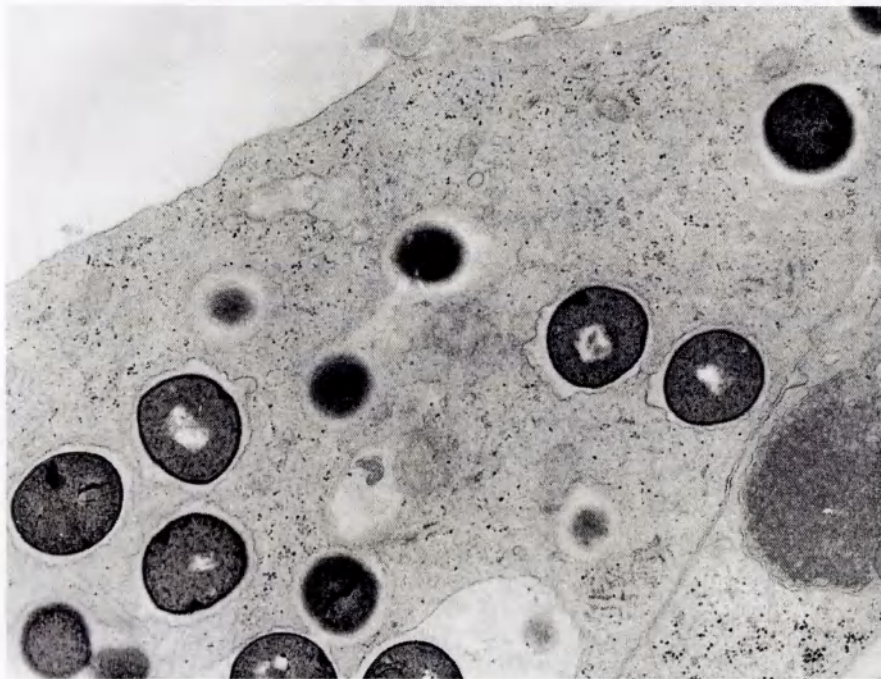
#### **2.4.5 Endothelial cell adherence assay examined by scanning and transmission electron microscopy.**

Scanning and transmission electron microscopy (SEM and TEM, respectively), of endothelial cell adherence assays were performed by Dr David Ferguson, Nuffield Department of Clinical Laboratory Sciences, University of Oxford. SEM was performed using endothelial cells on Thermanox coverslips following the adherence assay described above. This captures the assay with both adherent and internalized bacteria. Colour enhanced SEM views are shown in Figure 2.11. Bacteria appeared to be adherent to the monolayer, and were undergoing a process of uptake by the endothelial cell. Uptake appeared to be an active process, with endothelial cell projections (filaments and folds) seeming to envelop the bacteria. TEM was again performed following the standardised assay, with the exception that the monolayer was grown to confluence on Transwells. TEM views are shown in Figure 2.12. Bacteria can be seen both adherent to the endothelial cell surface, and in an intracellular location following uptake by the endothelial cell.





**Figure 2.11 Endothelial cell adherence assay examined by scanning electron microscopy.** Colour enhanced views at a magnification of X8,800 (top view), and X17,500 (bottom view).



**Figure 2.12 Endothelial cell adherence assay examined by transmission electron microscopy.** Magnification X2500 (top view), and X10,000 (bottom view).

#### **2.4.6 Endothelial cell internalization assay for *S. aureus*.**

Internalization of *S. aureus* by endothelial cells was evaluated by incubating the monolayer with lysostaphin at the end of the adherence assay. This has been shown to remove *S. aureus* adherent to the monolayer but does not affect the viability of internalized bacteria (Vann & Proctor, 1987). The endothelial cell adhesion assay was performed using the method described above, at the end of which coverslips were rinsed three times in M199 and placed into 24-well plates containing lysostaphin at 10µg/ml in M199. These were incubated for 20 minutes at 37°C in CO<sub>2</sub> then fixed and stained as before. Crystal violet penetrated the endothelial cell and stain internalized bacteria.

Internalization of *S. aureus* was also examined in a second assay in which 10<sup>8</sup> cfu were centrifuged onto the surface of the endothelium during a 5 minute spin at 1,000 rpm. The adherence assay was then allowed to continue over 1 hour at 37°C in CO<sub>2</sub>, followed by lysostaphin treatment as described above. Coverslips were rinsed, fixed, and stained, and the numbers of bacteria per mm<sup>2</sup> counted using the standardised counting procedure.

#### **2.4.7 Endothelial cell internalization assay for *L. lactis*.**

An alternative method was devised to measure invasion of endothelial cells by *L. lactis* expressing *S. aureus* adhesins, as the enzymes used to lyse external bacteria (mutanolysin and lysozyme) cause efficient bacterial cell lysis and death but do not result in effective bacterial removal from the endothelial cell surface, making visual counting unreliable. Sterile 24-well tissue culture plates were blocked with BSA (1%, v/v) for 1 hour and rinsed twice with PBS. A washed bacterial inoculum of 10<sup>8</sup> cfu/ml suspended in 500µl M199 was added to each well. Confluent endothelial cells on Thermanox coverslips were added after dip-washing three times in M199, and the plates were incubated at 37°C in CO<sub>2</sub> for 1 hour. The coverslips were rinsed three times in M199 and placed into 24-well plates containing gentamicin 200µg/ml and incubated at 37°C in CO<sub>2</sub> for 1

hour to kill extracellular bacteria. The coverslips were rinsed in PBS and the internalized bacteria released by incubating the coverslips in 500 $\mu$ l PBS containing 1% Triton X100 for 20 minutes at room temperature. Appropriate dilutions were plated onto M17 agar, and the number of colonies counted after overnight incubation at 30°C in air.

## **2.5 Methods used to evaluate cell wall-associated and secreted staphylococcal proteins.**

### **2.5.1 Quantitative determination of total protease activity in *S. aureus* culture supernatant.**

Isolates were inoculated from frozen stocks into 10ml of TSB and incubated for 15 to 18 hours under constant rotation at 37°C in air. The culture was centrifuged at 3,000 rpm for 10 minutes, and the supernatant concentrated 20 times using a Centricon-10 (Amicon). Quantitative determination of total protease activity in the 20-fold concentrated culture supernatants was performed using a protease assay kit according to the manufacturer's instructions (Calbiochem). In brief, fluorescein thiocarbamoyl-casein derivative (FTC-casein) was incubated with the sample for 24 hours at 37°C. Trichloroacetic acid (5%, w/v) was added to precipitate FTC-casein not cleaved into TCA-soluble peptides by protease activity. The sample was centrifuged and the absorbance of the supernatant measured at 492nm. A standard curve using V8 protease demonstrated that the intensity of colour was proportional to concentration.

### **2.5.2 Removal of surface proteins with proteinase K.**

Bacteria were incubated in TSB for 15 to 18 hours under constant rotation at 37°C in air then centrifuged at 3,000 rpm for 10 minutes. The bacterial pellet from a 10ml culture was resuspended in proteinase K (500 $\mu$ g in 0.5ml of 0.05M Tris pH 7.5) and incubated at 37°C for 45 minutes. Reactions were terminated by the addition of 30 $\mu$ l of the proteinase K inhibitor phenylmethylsulfonyl fluoride

(PMSF) at 4°C for 5 minutes, with centrifugation at 7,000 rpm for 10 minutes between and after treatments. The absence of residual enzymatic activity was confirmed using gelatin on the surface of an unprocessed Kodak X-Omat film as the substrate for proteolytic activity. A 50µl aliquot was applied onto a film which was incubated in a moist chamber for 1 hour at 37°C and then washed in running water. PBS was used as a negative control, and 50µl of bacterial suspension treated with proteinase K alone was used as a positive control. A clear halo on the film at the site of application was considered positive.

### **2.5.3 Preparation of cell wall-associated protein extracts.**

Overnight cultures of *S. aureus* were sub-cultured into 25mls of BHI to achieve an initial OD<sub>600</sub> of 0.1 and incubated with shaking at 37°C to an OD<sub>600</sub> of 0.8. Cells were harvested by centrifugation at 5,000 x g for 10 minutes, washed in PBS and adjusted to 10<sup>10</sup> cfu/ml in raffinose (30%, w/v) prepared in Tris-HCl (0.05M) pH 7.5 and NaCl (0.145M) supplemented with a mixture of proteinase inhibitors (Complete™, Boehringer Mannheim). Cell wall proteins were released from protoplasts by incubating with lysostaphin 100µg/ml (AMBI) at 37°C for 20 minutes. The lysate was centrifuged at 12,000 x g for 10 minutes, the supernatant containing extracted cell wall proteins being removed and stored at -80°C.

### **2.5.4 SDS-polyacrylamide gel electrophoresis (PAGE) and Western ligand affinity blotting or immunoblotting.**

Supernatant containing extracted cell wall proteins were boiled for 10 minutes in an equal volume of sodium dodecyl sulfate (SDS)-sample buffer (0.125 M Tris-HCl, pH 6.8; SDS 4%, w/v; glycerol 20%, v/v; β-mercaptoethanol 10%, v/v; and bromophenol blue 0.002%, w/v), and separated by SDS-PAGE using a 4.5% to 10% acrylamide gradient using standard methods (Laemmli, 1970). Proteins were transferred electrophoretically to PVDF Western blotting



membrane (Boehringer Mannheim) by the semi-dry transblot system (BioRad) in Tris-HCl pH 8.3 (48mM), glycine (39mM) and methanol (20%). Membranes were incubated for 15 hours at 4°C in 10mM Tris/HCl (pH 8.0), 500mM NaCl, 0.1% Tween 20 (TBST) containing BSA (2.5%, w/v).

Fibronectin-binding proteins were detected by ligand affinity blotting or immunoblotting. In the former, the membrane was incubated with pure human fibronectin (Chemicon Int.) 30µg/ml in TBST. After rinsing in TBST the membrane was incubated with monoclonal antibody Mab-1936 raised against the N-terminus of fibronectin (1:5000, Chemicon) followed by peroxidase-conjugated anti-mouse antibody (1:10,000, Amersham Int.). Detection was enhanced by chemiluminescence (Boehringer Mannheim) as instructed by the manufacturer. Immunoblotting was performed using polyclonal rabbit antibody to fibronectin-binding protein (1:2000, provided by Professor's M. Höök and M. McGavin), and alkaline-phosphatase conjugated protein A (1:2000) as the detection reagent.

Western immunoblotting was used to detect serine (V8) protease production by *S. aureus* using rabbit polyclonal anti-V8 protease antibody (1:2000, a gift from Dr S. Arvidson), followed by peroxidase-conjugated goat anti-rabbit immunoglobulin (1:2000, Dako).

## **2.6 Molecular biology techniques.**

### **2.6.1 Phage transduction.**

*Phage plate stock:* The bacterial strain containing the marker to be transduced was incubated with shaking overnight in THB at 37°C in air. A 20µl aliquot was diluted in 2ml of phage broth containing 10mM calcium chloride and incubated at 37°C in air with shaking for 4 hours. Phage 85 stock was diluted from 10<sup>-1</sup> to 10<sup>-5</sup> in phage broth containing 10mM calcium chloride, and 300µl of bacterial cells were added to 200µl of phage at each concentration. After 30 minutes at room temperature, 10ml of molten phage agar at 55°C containing 10mM calcium chloride was added and the mixture immediately poured over two

phage base plates. The plates were placed base down in a plastic bag to prevent drying and incubated at 37°C in air overnight, then examined visually to identify the plate with the highest dilution to give confluent lysis. The top layer was scraped off from this plate and centrifuged at 15,000 rpm for 10 minutes. The supernatant was filtered through a 0.45µm filter, checked for sterility by plating 10µl onto a blood agar plate which was incubated overnight at 37°C in air, and stored at 4°C.

*Phage transduction:* Recipient bacteria were grown overnight in 20ml of TSB in a 250ml flask with shaking at 37°C in air, centrifuged at 10,000 rpm for 10 minutes at 4°C and resuspended in 1ml of TSB. 500µl of bacterial cells were added to 1ml of LB containing 10mM calcium chloride and heated to 56°C in a water bath for 2 minutes, after which 1ml of phage containing the marker to be transduced was added. The control was 500µl bacterial cells and 1.5ml LB with calcium chloride minus phage. These were incubated at 37°C in a water bath for 25 minutes then transferred to ice where 1ml of ice cold 0.02M sodium citrate was added. This was spun at 10,000 rpm for 10 minutes at 4°C and the cells resuspended in 1ml of 0.02M sodium citrate and left on ice for 2 hours. Aliquots of 100µl were then spread onto TSA plates containing 0.05% sodium citrate and the appropriate antibiotic to select for the marker, and incubated for 48 hours at 37°C in air. Single colonies were plated to blood agar, and the presence of the transduced marker verified by PCR and phenotypic assay.

### **2.6.2 Competent cells.**

*E. coli:* Two millilitres of an overnight culture of DH5α or XL1-blue in LB media was added to 400 ml of fresh LB in a 2 litre glass flask and incubated at 37°C in air with shaking for 2-2.5 hours until the cells reached 0.4-0.5 at OD<sub>500</sub>. Cells were chilled on ice for 1 hour and spun at 4,000 x g for 10 minutes at 4°C. The pellet was resuspended in 30ml ice cold 100mM MgCl<sub>2</sub>, spun again as above, resuspended in 100ml ice cold 100mM CaCl<sub>2</sub> and placed on ice for an hour. Cells

were spun and resuspended in 20ml fresh CaCl<sub>2</sub> to which 3ml of glycerol (80%, v/v) was gradually added. Cells were aliquoted, snap frozen in liquid nitrogen and stored at -80°C until required.

*S. aureus*: Three millilitres of an overnight culture grown in TSB was added to 500ml of LB and incubated with shaking at 37°C in air to an OD<sub>450</sub> of 0.2. Cells were chilled on ice for 10 minutes and harvested by centrifugation at 3,000 x g for 10 minutes at 4°C. The pellet was washed twice with 50ml of ice cold 0.5M sucrose and finally resuspended in 1ml of the same solution. After maintaining the cells on ice for 20 minutes the cells were harvested and resuspended in 1ml ice cold 0.5M sucrose, aliquoted, snap frozen in liquid nitrogen and stored at -80°C until required.

*L. lactis*: An overnight culture was diluted 1:100 in M17 supplemented with 2.5% glycine and incubated at 30°C static for approximately 6 hours to reach 0.5-0.6 at OD<sub>600</sub>. The cells were then treated as described for *S. aureus* above.

### **2.6.3 Transformation and electroporation.**

*E. coli*: cells were transformed following CaCl<sub>2</sub> treatment (*Sambrook et al.*, 1989). In brief, 200µl of competent cells were added to 20µl of plasmid DNA and placed on ice for 30 minutes, then heat shocked at 42°C for 2 minutes. After a further 2 minutes on ice, 1ml of LB was added and the mixture incubated in a water bath at 37°C for 1 hour. Neat and concentrated suspension (100µl of each) was then plated onto Luria agar containing the appropriate antibiotic.

*S. aureus*: recombinant plasmids constructed in *E. coli* cannot be transformed into, or inherited stably by, wild type or even restriction-deficient mutants of *S. aureus* such as 879R4 or 80CR3 (Stobberingh & Winkler, 1977). Strain RN4220 is used as the first host for receiving chimaeric plasmids. This is defective in a restriction modification system but must carry an additional mutation that allows chimaeric plasmids transferred from *E. coli* to be inherited



stably. Once established in RN4220 the plasmids are easily transferred to other strains by electroporation or transduction.

The methodology used to transform *S. aureus* by electroporation was as follows. Electroporation was carried out using 200µl of competent cell suspension. Cells were mixed with 0.1 to 1.0µg of plasmid DNA and placed in a Gene Pulser cuvette with a 0.2cm electrode gap. The settings for transformation were voltage, 2.5kV; capacitor, 25µF; and resistance, 100ohms. After electroporation the cells were immediately placed in 300µl of TSB and incubated with shaking at 37°C in air, then plated onto TSA containing the appropriate antibiotic.

*L. lactis*: electroporation was carried out as above, except that electroporated cells were suspended in 1ml of M17, chilled on ice for 10 minutes then incubated at 30°C for 2 hours. These were plated onto M17 agar containing the appropriate antibiotic.

#### **2.6.4 Extraction of genomic DNA.**

Genomic DNA was extracted from *S. aureus* and coagulase-negative staphylococci using a genomic DNA purification kit using the manufacturer's instructions (Advanced Genetic Technologies Corp.), modified by the addition of lysostaphin 10U/ml (Ambi) to each sample at the lysis step.

#### **2.6.5 Manipulation of DNA.**

DNA manipulations were performed by standard procedures (Sambrook *et al.*, 1989). Restriction enzymes were purchased from New England BioLabs or from Boehringer Mannheim and were used as recommended by the suppliers. DNA amplified by PCR and used in cloning experiments was purified by Wizard® PCR Preps DNA purification system (Promega Corp.). Plasmid DNA for cloning was purified by WizardPlus® minipreps (Promega Corp.), with the addition of

either mutanolysin (500U/ml) and lysozyme (200µg/ml), or lysostaphin (10U/ml) in the cell resuspension buffer to lyse *L. lactis* and *S. aureus*, respectively.

#### **2.6.6 Polymerase chain reaction.**

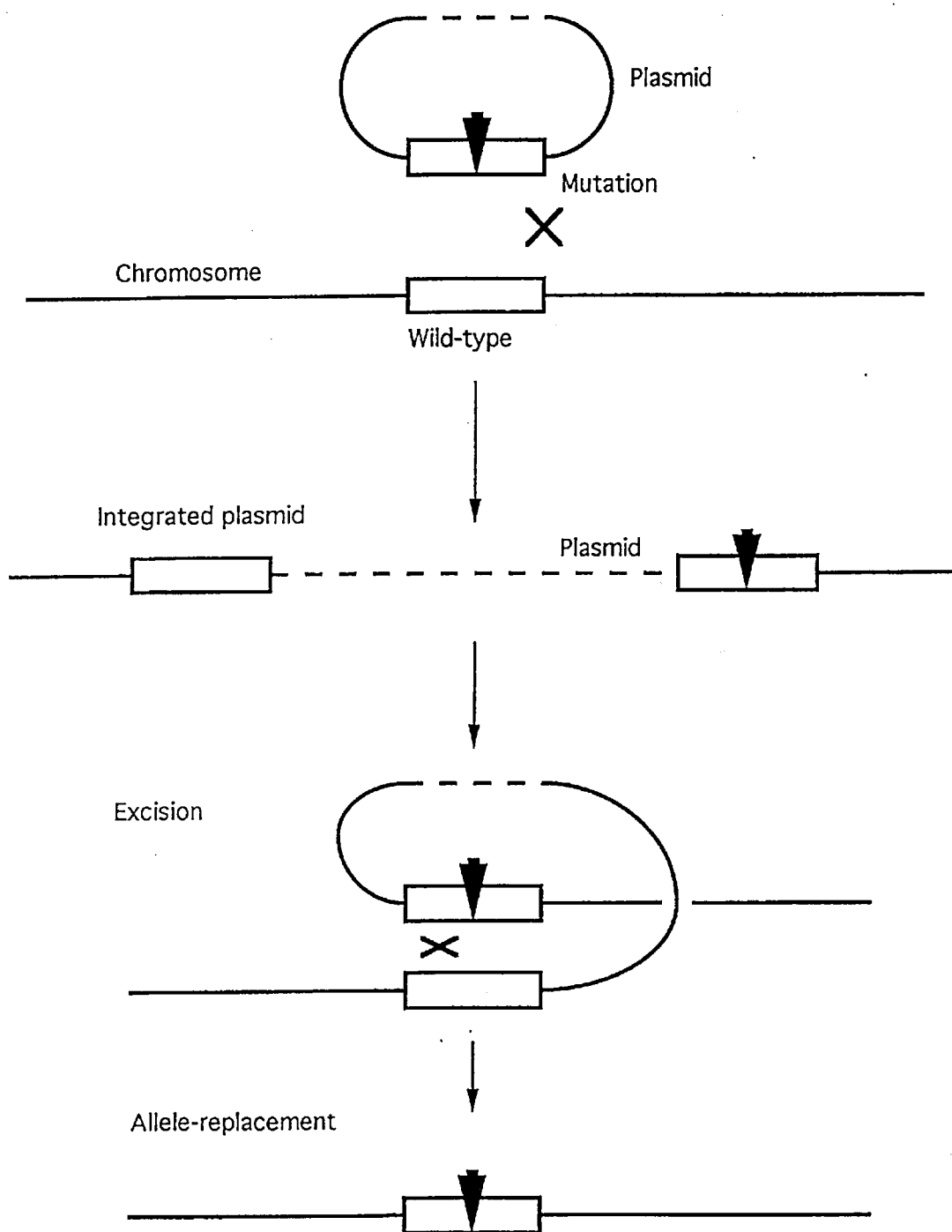
PCR amplifications were performed in a DNA thermal cycler (Perkin-Elmer Cetus) or a PTC-200 Peltier thermal cycler (MJ Research) using *Taq* or *Pfu* polymerase (Boehringer Mannheim). Final concentrations of PCR reaction components were 1X PCR buffer, 0.2mM of each dNTP, variable magnesium (detailed in later chapters), 100pmol of forward and reverse primers, template DNA and 2.5U units of polymerase in a 100µl volume. Cycling parameters are provided in the relevant chapters.

#### **2.6.7 Southern hybridization.**

Probes were made using PCR based DIG-labelling. DNA fragments were generated by PCR amplification using sequences in the GenBank database, details of which are given in subsequent chapters. Aliquots of the PCR reaction mixtures were analyzed by 1% agarose gel electrophoresis, purified by means of a DNA extraction kit (Boehringer Mannheim) and DIG-dUTP labelled by PCR using the method recommended by the suppliers (Boehringer Mannheim). DNA hybridization was otherwise performed by the method of Southern (Southern, 1975). In brief, genomic DNA was digested with one or more restriction enzymes and run on a 1.5% agarose gel. The DNA was depurinated, denatured, neutralized and then transferred onto a nylon membrane by capillary transfer. DNA was fixed by baking at 80°C for 10 minutes. Membranes were prehybridized for 4 hours at 68°C, then hybridized overnight using 1.5µl/ml of probe. Membranes were then washed and developed using anti-DIG antibody and chemoluminescent detection. Where dot blots were used, 2µl of genomic DNA was spotted onto nylon membrane, fixed by baking and hybridized as above.

#### **2.6.8 Constructing a defective bacterial mutant using allelic replacement.**

Allelic replacement by recombination allows a mutation that has been deliberately constructed in a cloned gene to be introduced into the chromosome by homologous recombination. This approach was used to construct a *S. aureus* mutant defective in serine (V8) protease. Details are provided in chapter 5, but the principles on which this was based are shown in Figure 2.13.



**Figure 2.13. Plasmid integration and excision.** The plasmid integrates into the chromosomal locus by a single cross-over event, and subsequently excises by a recombination event on the opposite side of the chromosomal locus. The wild type gene is thus replaced by the mutant copy.

### **2.6.9 Protein constructs and their expression by *S. aureus* and *L. lactis*.**

Construction of plasmids encoding one or more regions of *S. aureus* fibronectin-binding protein A and protein expression by *S. aureus* and *L. lactis* was used extensively in chapter 3 where it is described in detail.

### **2.7 Statistical analysis.**

Results for the numbers of bacteria adherent to purified fibronectin where visual counting was used, or adherent to or internalized by endothelium, were expressed as the mean count per 1mm<sup>2</sup> surface area. All points were performed in triplicate or quadruplicate and each experiment was performed three times. Statistical analysis was carried out using the Statview 4.5 statistical software package (Abacus). Comparison of the mean count between bacterial strains was performed using a t-test. For the non-visual microtitre plate adherence assay, all points were performed in quadruplicate and each experiment was performed three times. Comparison was then made between the mean optical density. Other statistical tests used are described in subsequent chapters.

## **Chapter 3. Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence and invasion of human endothelial cells by *S. aureus*.**

### **3.1 Chapter content.**

The bacterial determinants that promote adherence and invasion of endothelium by *S. aureus in vitro* had not been elucidated prior to the start of this work. The aim of this study was to evaluate the role of a number of bacterial surface structures in this process. The basic approach taken was to compare the adherence of a range of defective bacterial mutants with that of the isogenic parent. The bacterial determinants chosen were those known to interact with human soluble and/or matrix proteins at the beginning of the thesis. The experimental system used to determine adherence and subsequent bacterial uptake was a standardised endothelial cell assay, as described in chapter 2.

### **3.2 Bacterial strains.**

The laboratory strains of *S. aureus* used, and their sources, are listed in Table 1. The clinical *S. aureus* strains JR75, JR76, JR77, JR78, and JR80 were isolated in the Oxford microbiology department in 1995 from blood cultures of patients with native valve endocarditis. Phillips and a mutant strain of Phillips defective in collagen-binding protein (PH100) were gifts from Drs. Magnus Höök and Jo Patti, Texas. The *fnbA*::Tc<sup>R</sup> *fnbB*::Em<sup>R</sup> mutations (the double mutation for genes encoding fibronectin-binding proteins A and B) were co-transduced from DU5883 to strains P1 and JR80 by phage 85 mediated transduction (Asheshov, 1966), to construct strains DU5947 and DU5953, respectively. Transductants resistant to tetracycline 2µg/ml and erythromycin 10µg/ml were selected. The lack of adherence to purified human fibronectin 10µg/ml was confirmed by microtitre adherence assay.

**Table 3.1 Strains of *S. aureus* used in this study.**

<b>Strain</b>	<b>Relevant genotype</b>	<b>Properties</b>	<b>Reference or source</b>
<i>S. aureus</i> 8325-4		NCTC 8325 cured of prophages	(Novick, 1967)
DU5883	<i>fnbA::Tc<sup>R</sup> fnbB::Em<sup>R</sup></i>	Mutant strain of 8325-4 defective in fibronectin-binding proteins A and B	(Greene <i>et al.</i> , 1995)
DU5883 (pFNBA4)	<i>fnbA::Tc<sup>R</sup> fnbB::Em<sup>R</sup> (pFNBA4:fnbA<sup>+</sup> Cm<sup>R</sup>)</i>	Mutant strain of 8325-4 defective in fibronectin-binding proteins, complemented with multicopy plasmid expressing <i>fnbA</i> <sup>+</sup>	(Greene <i>et al.</i> , 1995)
DU5883 (pFNBB4)	<i>fnbA::Tc<sup>R</sup> fnbB::Em<sup>R</sup> (pFNBB4:fnbB<sup>+</sup> Cm<sup>R</sup>)</i>	Mutant strain of 8325-4 defective in fibronectin-binding proteins, complemented with multicopy plasmid expressing <i>fnbB</i> <sup>+</sup>	(Greene <i>et al.</i> , 1995)
DU5880	<i>clfA::Tn917(Em<sup>R</sup>)</i>	Mutant strain of 8325-4 defective in fibrinogen-binding protein ClfA	(McDevitt <i>et al.</i> , 1994)
DU5857	<i>Δcoa::Em<sup>R</sup></i>	Mutant strain of 8325-4 defective in coagulase	(Phonimdaeng <i>et al.</i> , 1990)
DU5875	<i>Δspa::Tc<sup>R</sup></i>	Mutant strain of 8325-4 defective in protein A	(Patel <i>et al.</i> , 1987)
Phillips		Clinical osteomyelitis isolate expressing collagen-binding protein	(Patti <i>et al.</i> , 1994b)
PH100	<i>cna::Gm<sup>R</sup></i>	Mutant strain of Phillips defective in collagen-binding protein	(Patti <i>et al.</i> , 1994b)
Newman		High level of fibrinogen-binding protein ClfA	(Duthie & Lorenz, 1952)
DU5917	<i>cps::Tn917 (Em<sup>R</sup>)</i>	Mutant strain of Newman defective in capsular polysaccharide	(Sau <i>et al.</i> , 1997)
P1		Isolated from a rabbit inoculated with ATCC 25923	(Sherertz <i>et al.</i> , 1993)
DU5947	<i>fnbA::Tc<sup>R</sup> fnbB::Em<sup>R</sup></i>	Mutant strain of P1 defective in fibronectin-binding proteins A and B	This study
DU5908	<i>clfA::Tn917(Em<sup>R</sup>)</i>	Mutant strain of P1 defective in fibrinogen-binding protein ClfA	TJ Foster
JR80		Isolate from a patient with endocarditis	This study
DU5953	<i>fnbA::Tc<sup>R</sup> fnbB::Em<sup>R</sup></i>	Mutant strain of JR80 defective in fibronectin-binding proteins A and B	This study

Em, erythromycin; Cm, chloramphenicol; Tc, tetracycline; Gm, gentamicin.

### **3.3 Results.**

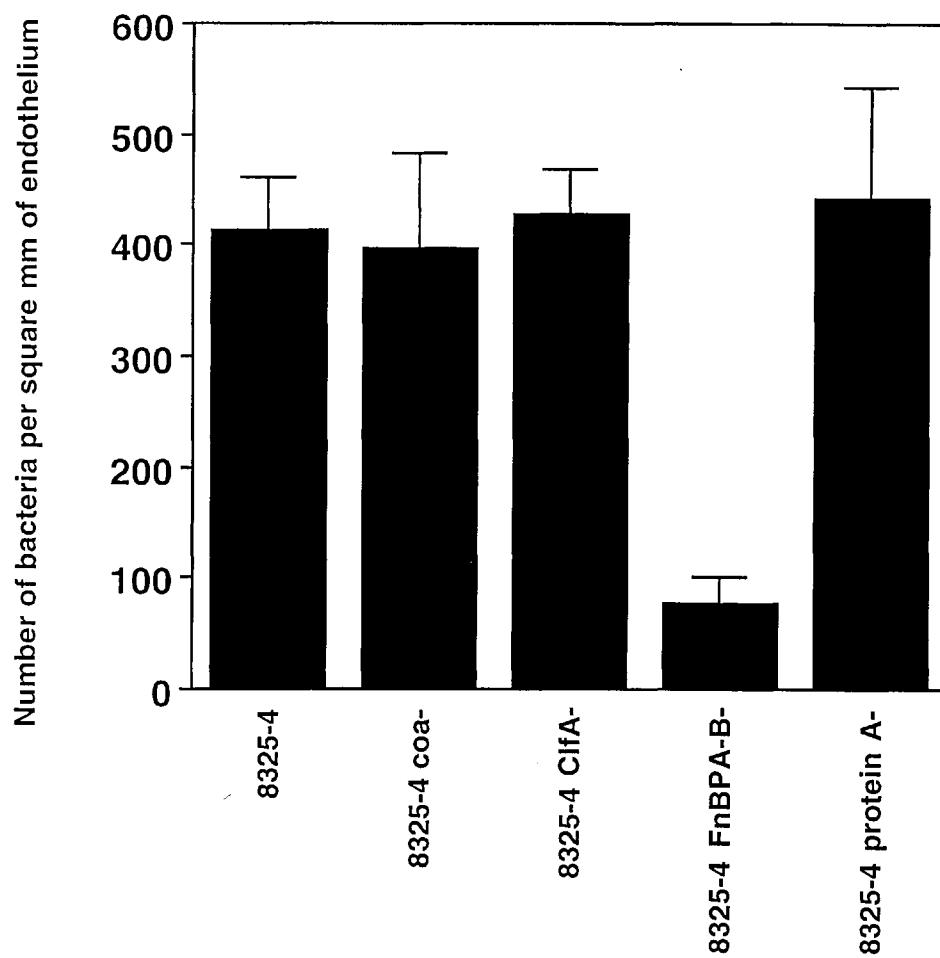
Experimental results for endothelial cell assays are shown throughout as the mean of triplicate experiments  $\pm$  standard error of the mean.

#### **3.3.1 Isogenic mutants deficient in fibronectin-binding protein demonstrate reduced adherence to endothelial cells.**

Adhesion to endothelium was compared for the isogenic mutants and parental strains shown in Table 1. Evaluation of 8325-4 and related mutants demonstrated a significant reduction in the number of adherent bacteria for the 8325-4 mutant deficient in FnBPA and FnBPB ( $P = 0.0008$ , Fig. 3.1). This contrasted with 8325-4 mutants defective in coagulase, protein A or fibrinogen-binding protein ClfA, for which adhesion was unaffected ( $P > 0.05$ , Fig. 3.1).

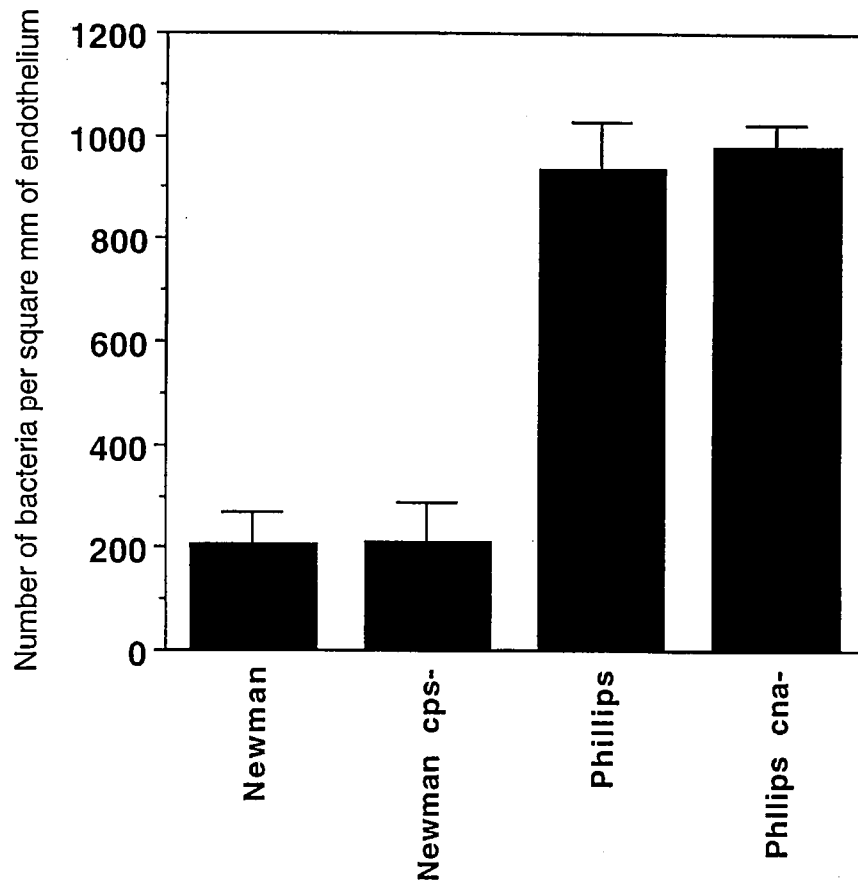
There was no difference between strain Newman and its isogenic mutant defective in capsular polysaccharide ( $P > 0.05$ , Fig. 3.2), or strain Phillips and its isogenic mutant defective in collagen-binding protein ( $P > 0.05$ , Fig. 3.2). Reduction in adherence of the 8325-4 FnBP-deficient mutant was reproducible using FnBP-deficient mutants of strains P1 and JR80 whose adherence was 9.2% and 14.7% respectively, compared with that for the parent strains ( $P < 0.0001$  in both cases, Fig 3.3).





**Figure 3.1 Adherence of *S. aureus* mutants to human endothelial cells *in vitro*.**

Isogenic mutants of 8325-4 were defective in coagulase (coa), the fibrinogen-binding protein ClfA, fibronectin-binding proteins FnBPA and FnBPB, or protein A.



**Figure 3.2 Adherence of *S. aureus* mutants to human endothelial cells *in vitro*.**

Isogenic mutants of Newman and Phillips were defective in capsular polysaccharide (cps) and collagen-binding protein (cna), respectively.

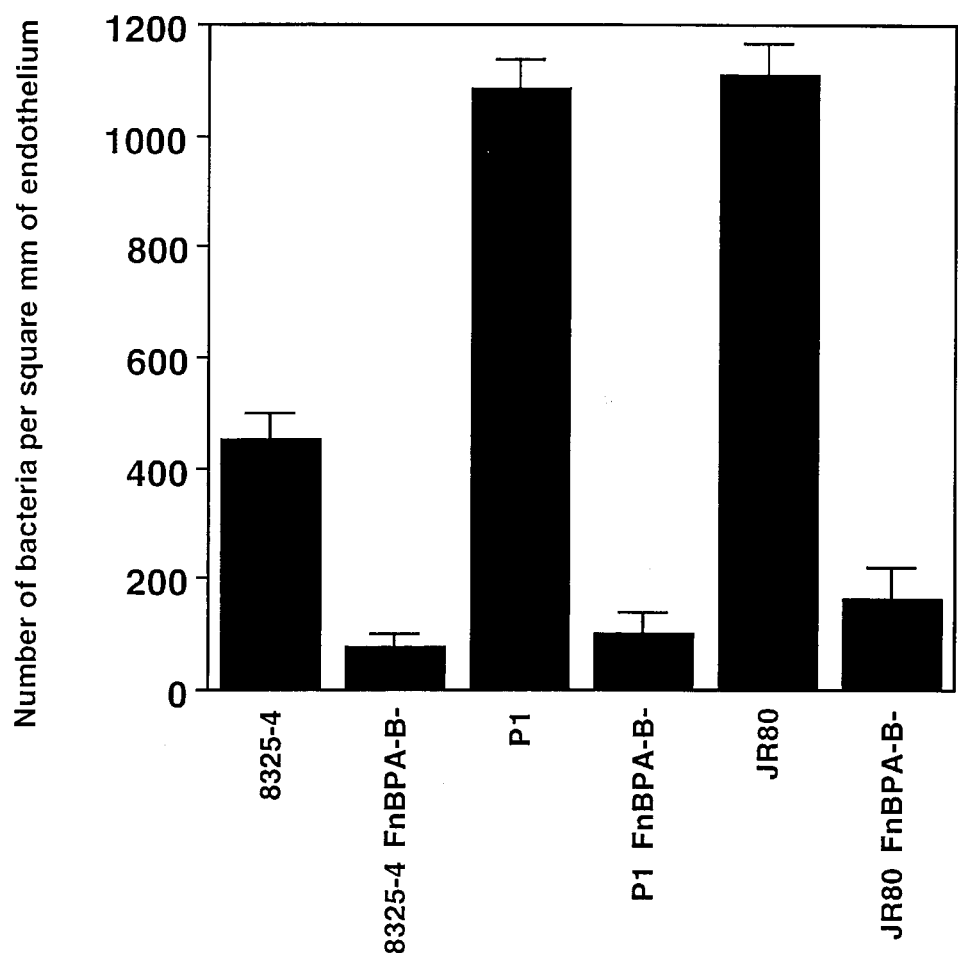
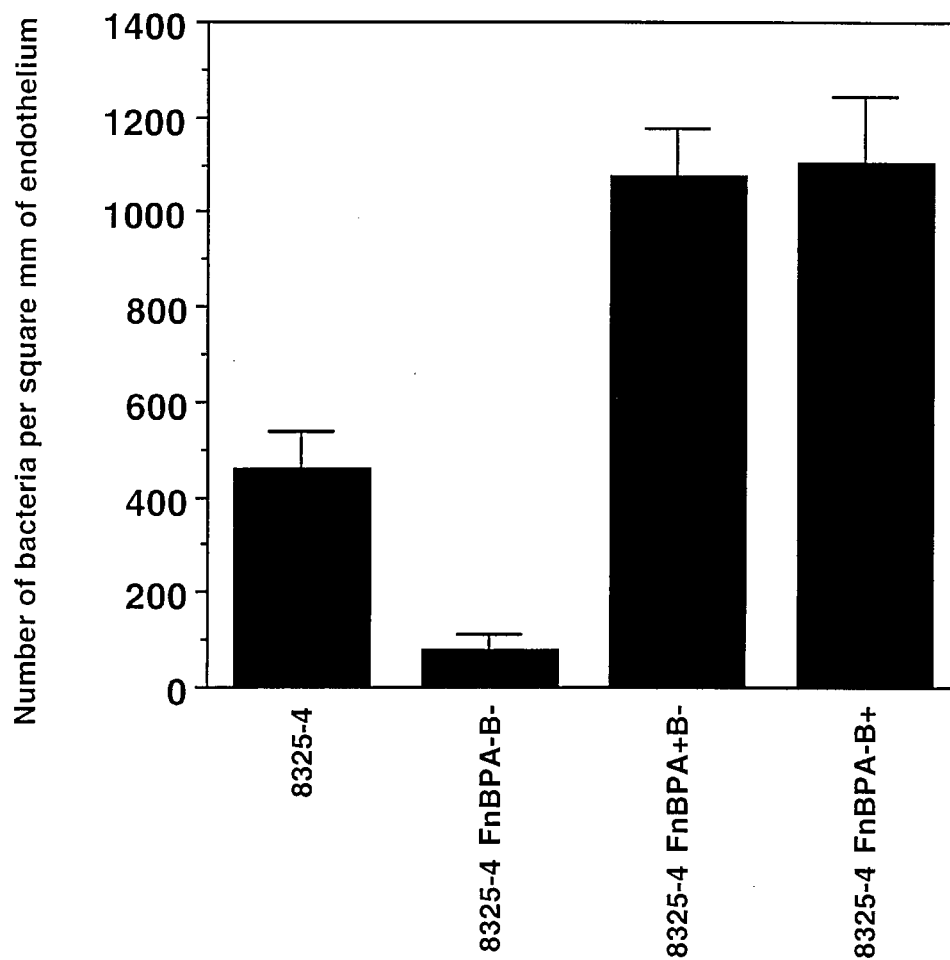


Figure 3.3 Adherence of FnBP-defective *S. aureus* mutants to human endothelial cells *in vitro*. Isogenic mutants with three different genetic backgrounds defective in fibronectin-binding proteins FnBPA and FnBPB.

### **3.3.2 Complementation of 8325-4 FnBP-deficient mutant with a multicopy plasmid carrying an *fnb* gene restores bacterial adherence to endothelial cells.**

Further evidence for the involvement of *S. aureus* FnBP in bacterial adherence to endothelial cells was provided by adhesion assays using the 8325-4 FnBP-deficient mutant complemented with a multicopy plasmid carrying either *fnbA* (pFNBA4) or *fnbB* (pFNBB4). Adherence was fully restored, both complemented strains demonstrating significantly greater levels of adhesion than that of the FnBP-deficient mutant ( $P < 0.0001$  and  $P = 0.0003$ , respectively), and wild type ( $P = 0.0012$  and  $0.0024$ , respectively), (Fig. 3.4).



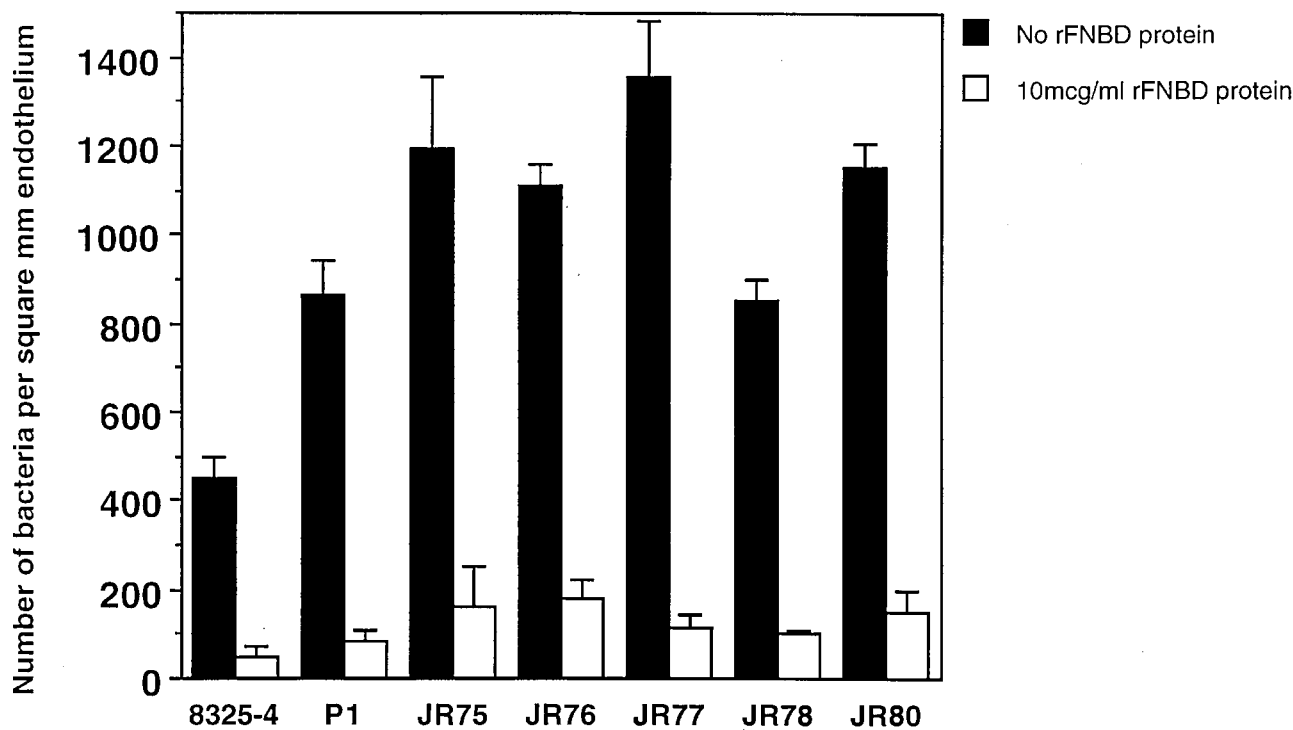
**Figure 3.4 Effect of complementation of FnBP-defective *S. aureus* mutants with *fnbA* or *fnbB* on adherence to endothelial cells *in vitro*.** *S. aureus* 8325-4 FnBPA<sup>+</sup>B<sup>-</sup> is the double FnBP mutant complemented with pFNBA4 encoding *fnbA*, and 8325-4 FnBPA<sup>-</sup>B<sup>+</sup> is the double FnBP mutant complemented with pFNBB4 encoding *fnbB*.

### 3.3.3 The recombinant form of the ligand-binding region of *Streptococcus dysgalactiae* FnBPB inhibits adherence of *S. aureus* to endothelial cells.

The involvement of region D of *S. aureus* FnBP (a region with ligand binding activity) in the interaction between this organism and human endothelial cells was evaluated by competitive adherence inhibition assays using the recombinant form of the binding region of FnBPB from *Streptococcus dysgalactiae* (rFNBD-B, a gift from Dr. Magnus Höök, Texas, referred to below as rFNBD protein). This protein has been shown to inhibit the binding of <sup>125</sup>I-labelled intact fibronectin or the N-terminal fibronectin domain to *S. aureus* (Joh *et al.*, 1994). Endothelial cell adhesion assays were performed using the method described in chapter 2, with the exception that rFNBD protein was added to the wells at a final concentration of 1µg/ml, 10µg/ml or 50µg/ml immediately prior to bacterial inoculation. To control for the possibility that recombinant protein non-specifically interfered with bacterial adherence, parallel assays were performed in the presence of a recombinant truncated ClfA protein (Clf41, residues 221-559) (O'Connell *et al.*, 1998) at a final concentration of 25µg/ml. A second control was bacteria in the absence of recombinant protein. The total volume was maintained at 500µl for all wells.

The effect of 10µg/ml rFNBD protein on adherence of *S. aureus* 8325-4, P1 and five recent clinical isolates is shown in Fig. 3.5. The presence of rFNBD protein resulted in a significant reduction in adherence for all Isolates, ( $P < 0.0001$ ), while the recombinant truncated ClfA protein (Clf41, residues 221-559) had no effect on adherence (range 89.6%-95.5% of the control,  $P > 0.05$ ) (data not shown). These results imply that the D region of *S. aureus* FnBP participates in the interaction between *S. aureus* and the endothelial monolayer. The Inhibitory effects were comparable for recombinant protein concentrations of 1µg/ml, 10µg/ml and 50µg/ml (Fig. 3.6). However, adheslon of 8325-4 and JR80 to the monolayer was not completely abolished at any of the concentrations of rFNBD protein used (Fig. 3.6). This concurs with the observation that FnBP

defective mutants can adhere to the monolayer, albeit at a reduced level compared to wild type.



**Figure 3.5** Effect of the recombinant form of the ligand-binding region of FnBPB from *Streptococcus dysgalactiae* on adherence of *S. aureus* to endothelial cells *in vitro*. Adherence assays were performed in the absence or presence of 10µg/ml of the recombinant form of the ligand-binding region of FnBPB encoded by *fnbB* of *Streptococcus dysgalactiae*.

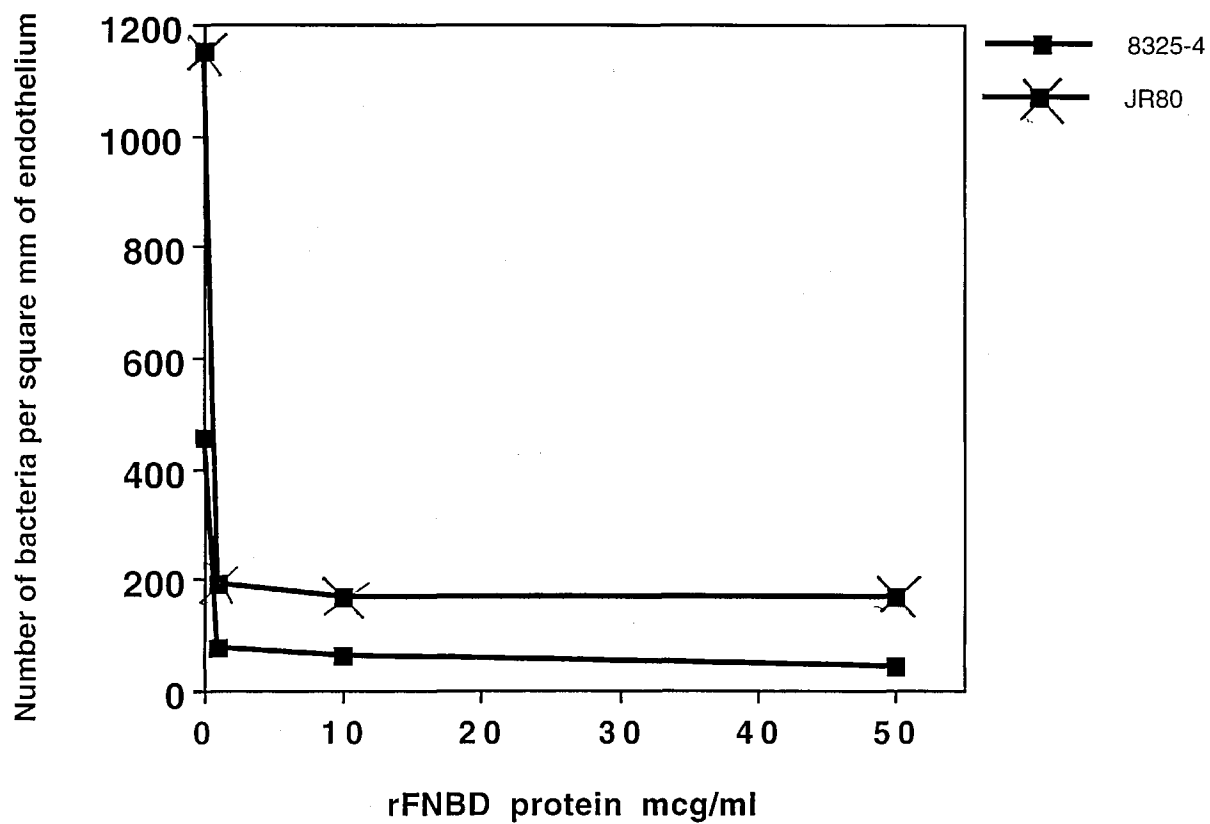


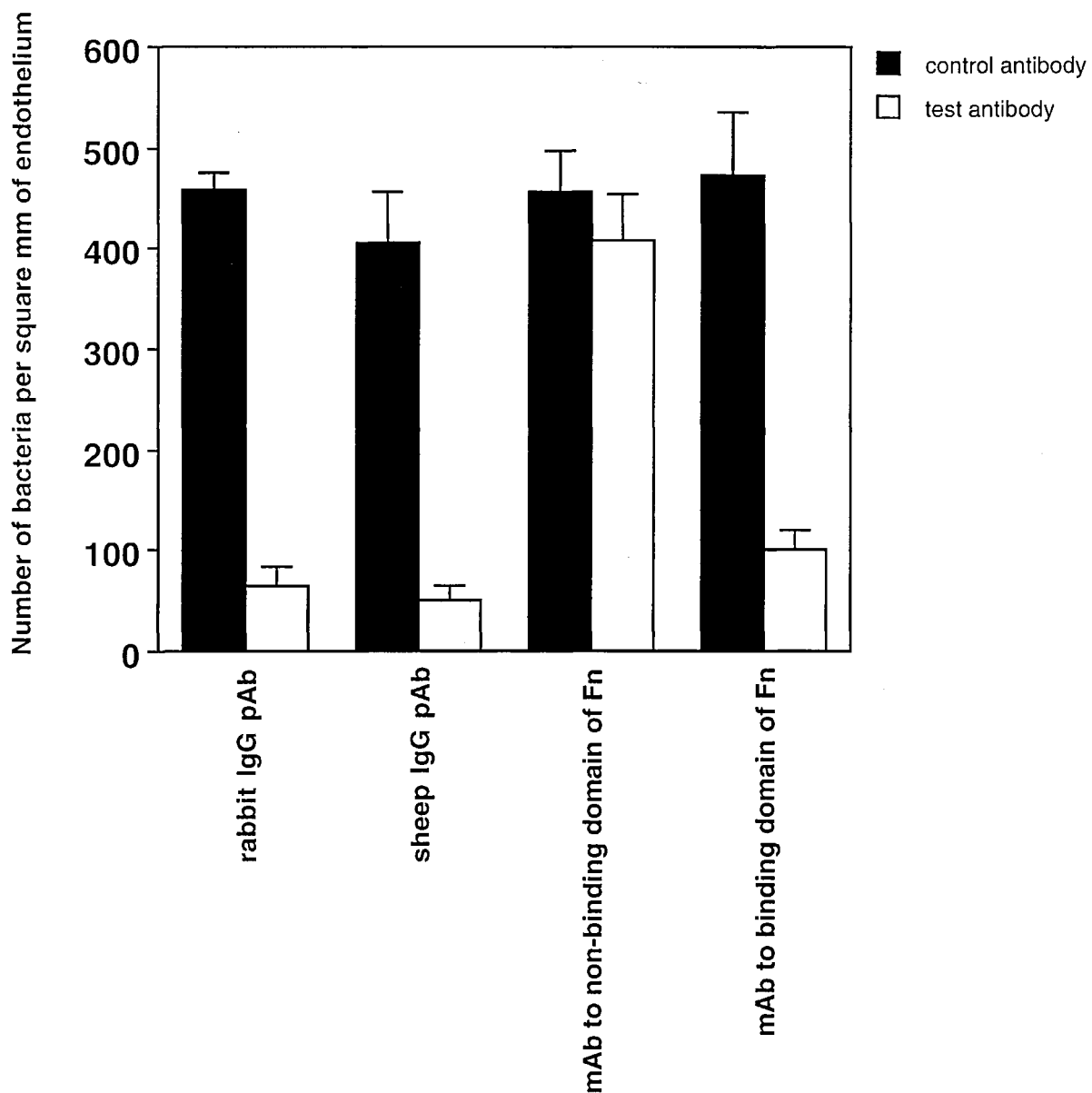
Figure 3.6 Effect of variable concentration of the recombinant form of the ligand-binding region of FnBPB from *Streptococcus dysgalactiae* on adherence of *S. aureus* to endothelial cells *in vitro*.



### **3.3.4 Anti-fibronectin antibodies inhibit bacterial adherence to endothelial cells.**

The above findings suggest that fibronectin is the host cell surface receptor for adherence of *S. aureus* to endothelial cells *in vitro*. This was further evaluated by examining the effect of a panel of anti-human fibronectin antibodies on adherence of 8325-4 defective in protein A (DU5875). This mutant was used to control for the confounding interaction between protein A and the Fc region of IgG. The following antibodies were used at a concentration of 10µg/ml: rabbit polyclonal antibody to purified human fibronectin (F3648, Sigma); sheep polyclonal antibody to purified human fibronectin (Serotec UK, Oxford); monoclonal antibody recognising an epitope located within the 5th type III repeat of human plasma fibronectin, (mouse IgG1, clone IST-4, F0916, Sigma); and monoclonal antibody recognising the N-terminus of fibronectin (mouse IgG3 clone 10B7, Biogenesis Ltd, Poole, UK). The following control antibodies were used at a concentration of 10µg/ml: normal sheep IgG (I 5131, Sigma); normal rabbit IgG (I 5006, Sigma); mouse IgG1, Kappa (MOPC-31c, M9035, Sigma); and mouse IgG3, Kappa (FLOPC-21, M3645, Sigma). All antibodies were added to the wells immediately prior to bacterial inoculation.

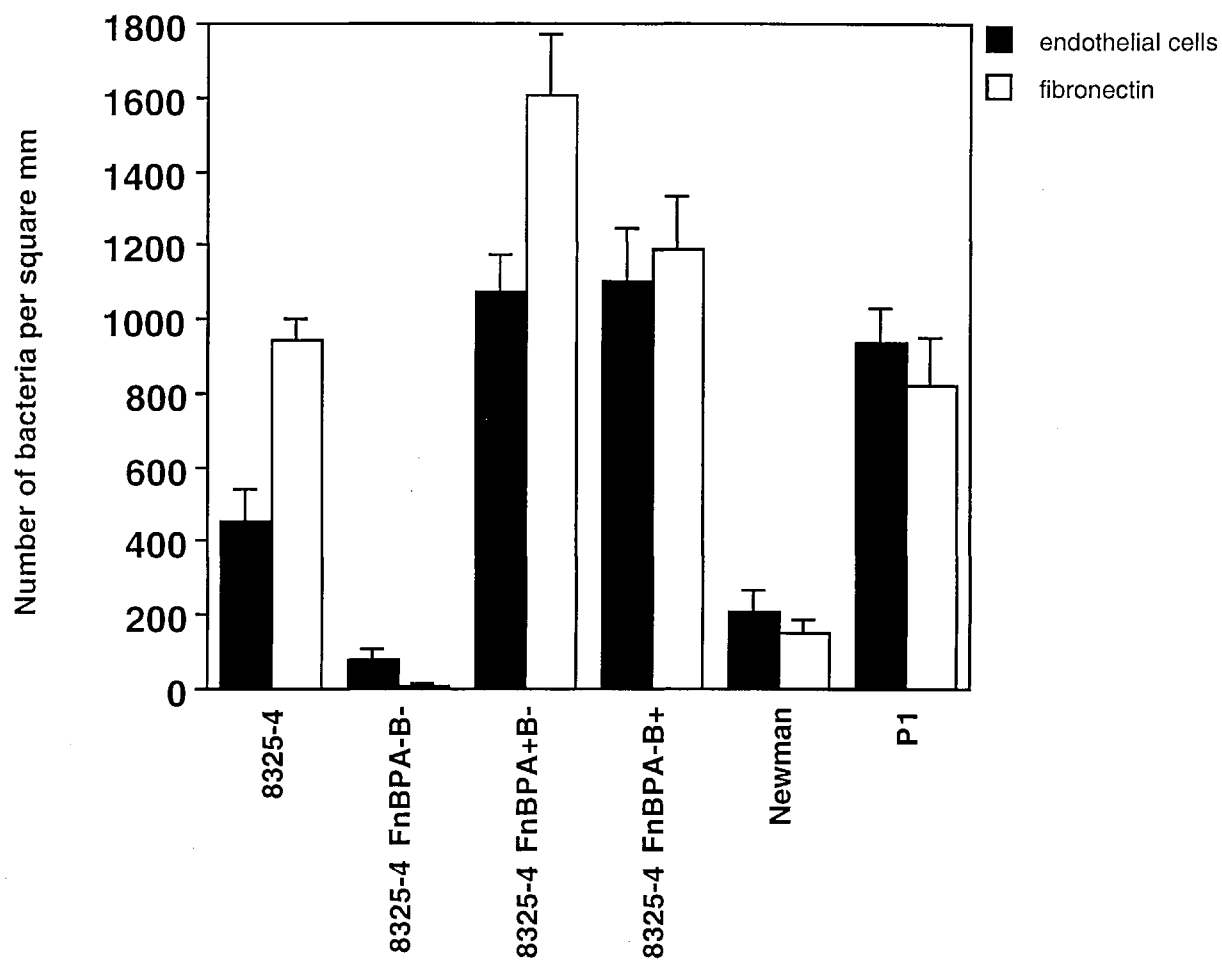
A significant reduction in adhesion was seen in the presence of rabbit or sheep polyclonal anti-human fibronectin antibodies ( $P = 0.0004$  in both cases), or a monoclonal antibody to the N-terminus of human fibronectin ( $P = 0.0008$ , Fig. 3.7). A monoclonal antibody recognising the 5th type III repeat of human fibronectin had no significant effect.



**Figure 3.7** The effect of anti-human fibronectin antibodies on the adherence of a protein A deficient mutant of 8325-4 to human endothelial cells *in vitro*.

### **3.3.5 Adherence of *S. aureus* to purified fibronectin is associated with adherence to endothelial cells.**

Adherence to endothelial cells was compared with adherence to solid-phase purified human fibronectin for 8325-4 and related fibronectin-binding protein mutants, P1, JR80 and Newman. Bacterial adherence to purified human fibronectin was evaluated using a visual counting method as described in chapter 2. Adhesion to endothelial cells was associated with adhesion to purified fibronectin (Fig. 3.8). As predicted, the 8325-4 FnBP-deficient mutant showed no binding to purified fibronectin. The same mutant complemented with a multicopy plasmid expressing either *fnbA* (pFNBA4) or *fnbB* (pFNBB4) showed enhanced binding to fibronectin compared with the isogenic parent. This enhancement has been reported previously (Greene *et al.*, 1995), and may be due to the surface expression of higher number of FnBP's. *S. aureus* P1 adhered strongly to endothelial cells and fibronectin, while Newman adhered poorly to both substrates. The weak adherence of Newman to solid-phase fibronectin in this study has been observed by others (Vaudaux *et al.*, 1995), and occurs despite the presence of two apparently functional *fnb* genes that express FnBP protein detectable by ligand-affinity blotting (Greene *et al.*, 1995). The association between the ability to adhere to solid-phase purified human fibronectin and endothelial cells provides further indirect evidence for fibronectin as the endothelial cell receptor for *S. aureus*.



**Figure 3.8 Association between adherence of *S. aureus* to purified fibronectin and endothelial cells *in vitro*.**

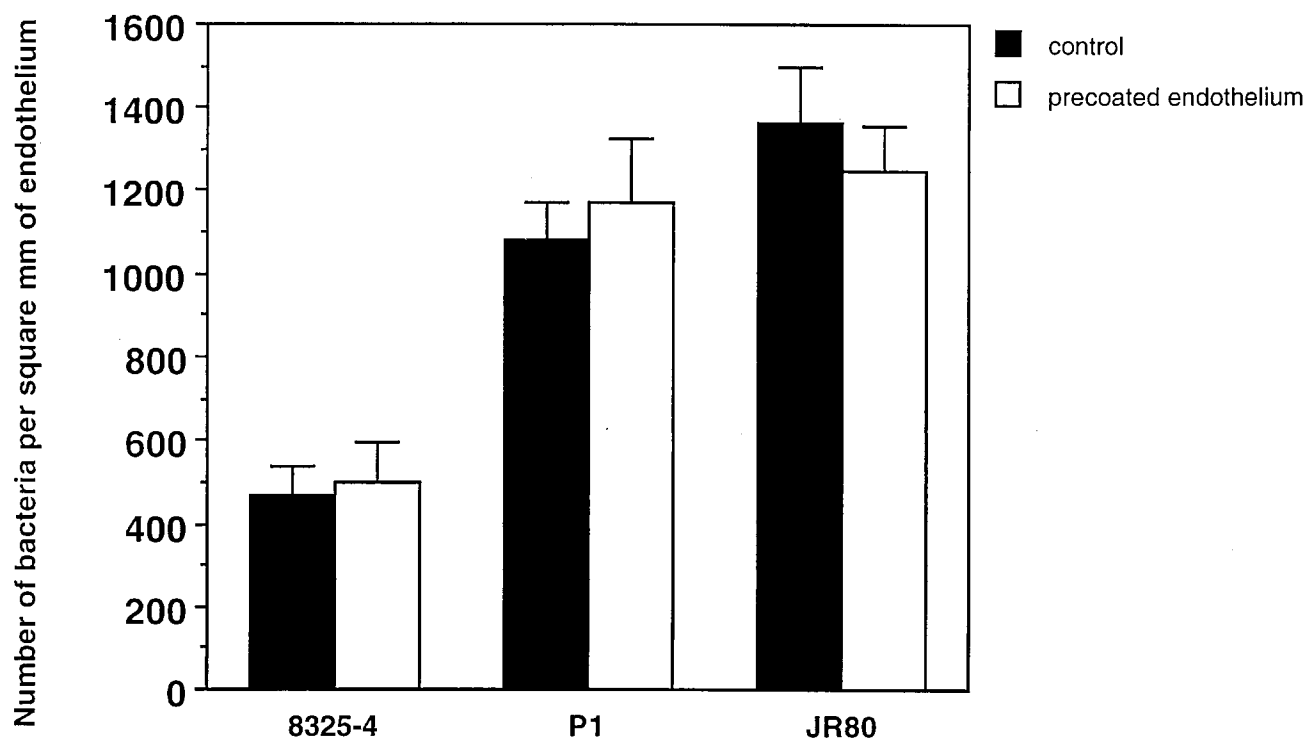
### 3.3.6 Effect of plasma proteins on adherence of *S. aureus* to endothelial cells.

The effect of plasma proteins was evaluated by pre-coating endothelial monolayers with purified human fibronectin or fibrinogen, or by adding these host proteins into the assay. Adherence assays were performed using one of two modifications:

- (1) Confluent endothelial cells coating Thermanox coverslips were pre-coated for 30 minutes at 37°C in air with either purified human fibronectin at 300µg/ml (Sigma), or purified human fibrinogen at 1mg/ml (Sigma). The coverslips were then dip-rinsed in M199 four times prior to use in the adherence assay.
- (2) Adherence assays were performed in the presence of either purified human fibronectin at 300µg/ml or purified fibrinogen at 1mg/ml, with or without rFNBD protein.

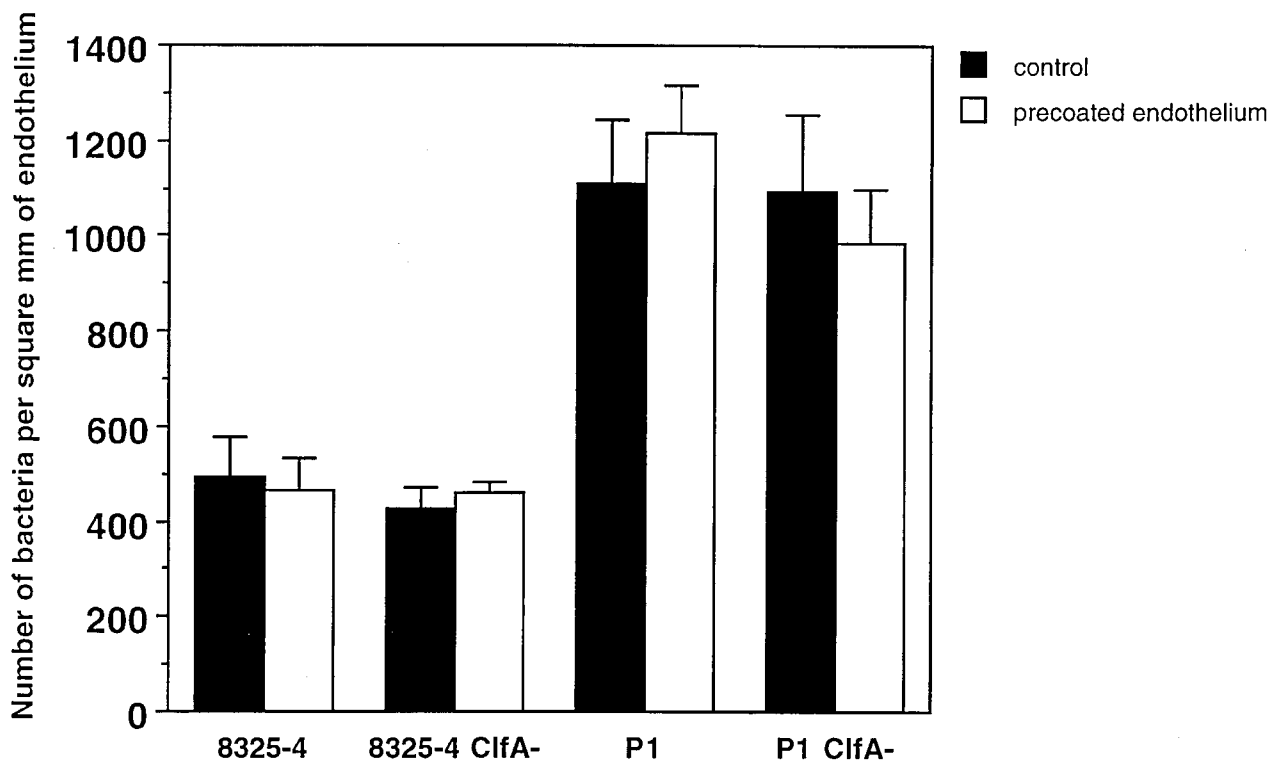
Contamination of commercially available human fibrinogen by fibronectin is common, and is a potential confounder in these assays. The human fibrinogen used in this study was therefore evaluated and purified prior to use, as follows. The presence of contaminating fibronectin was confirmed by Western immunoblotting following fractionation by SDS-PAGE of a 15µl aliquot of 2mg/ml fibrinogen solution dissolved in M199, as previously described (McDevitt *et al.*, 1992). Fibronectin was removed by passing the fibrinogen solution through a gelatin-Sepharose column (Pharmacia Biotech) as instructed by the manufacturer. The absence of contaminating fibronectin in the eluate was confirmed by Western immunoblot. Western immunoblotting did not demonstrate the presence of contaminating immunoglobulin.

Pre-coating the monolayer with fibronectin followed by rinsing prior to use in the adherence assay had no effect on bacterial adherence (Fig. 3.9). This contrasted with the effect of adding fibronectin at the start of the adherence assay, which led to agglutination of bacteria and adherence of large aggregates to the monolayer.



**Figure 3.9 Adherence of *S. aureus* to endothelial cells *in vitro* pre-coated with fibronectin.** Endothelial monolayers were pre-incubated with purified human fibronectin at 300µg/ml for 30 minutes followed by rinsing prior to use in the bacterial adherence assay.

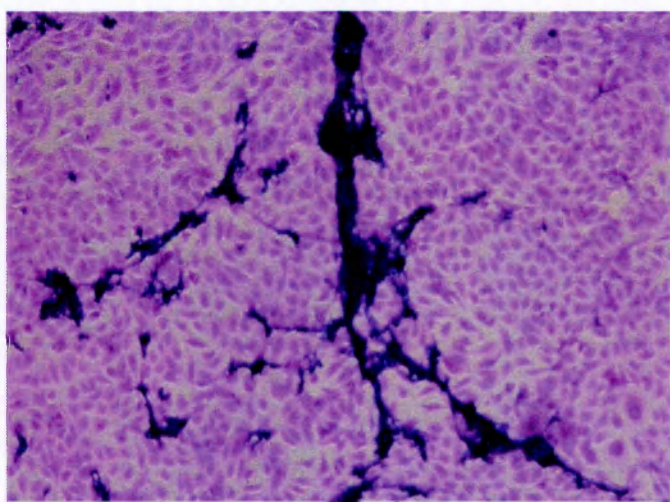
Precoating the endothelial monolayer with fibrinogen also failed to influence adherence of 8325-4 or P1 (Fig. 3.10). The adherence assay was repeated for mutants of 8325-4 and P1 defective in ClfA. There was no significant difference in adherence to fibrinogen pre-coated endothelium between these two pairs (Fig. 3.10).



**Figure 3.10 Adherence of *S. aureus* to endothelial cells *in vitro* pre-coated with fibrinogen.** Endothelial monolayers were pre-incubated with purified human fibrinogen at 1mg/ml for 30 minutes followed by rinsing prior to use in the bacterial adherence assay. 8325-4 ClfA<sup>-</sup> and P1 ClfA<sup>-</sup> are mutants defective in the fibrinogen-binding protein ClfA.



The relative importance of FnBP and ClfA in the adherence of *S. aureus* to endothelial cells *in vitro* in the presence of fibrinogen was then assessed by assays in which fibrinogen (1mg/ml) was added, with or without rFNBD protein (10µg/ml). This was performed for 8325-4, P1 and the 5 clinical isolates (JR75-78 and JR80), which all behaved similarly. Fibrinogen alone resulted in the formation of a latticework of bacterial aggregates adherent to the monolayer (Fig 3.11). The entire inoculum appeared to become consumed into the aggregates, with virtually no bacteria adherent to the monolayer in singles, pairs or small clusters. In the presence of rFNBD protein and fibrinogen together, large bacterial aggregates formed but these were not adherent to the monolayer and were readily rinsed off at the end of the assay. Thus, *S. aureus* fibronectin-binding proteins appear to predominate in importance over the fibrinogen-binding protein ClfA during bacterial adherence to endothelial cells in the presence of fibrinogen.



**Figure 3.11 Effect of fibrinogen on adherence of *S. aureus* 8325-4 to endothelial cells *in vitro*.** View under light microscopy using x10 magnification. Human fibrinogen 1mg/ml was added to the bacterial adherence assay.

### **3.3.7 *S. aureus* mutants deficient in fibronectin-binding protein do not invade endothelial cells *in vitro*.**

The number of bacteria internalized by endothelial cells was compared between wild type and FnBP-deficient mutants using the standardised adherence assay followed by incubation with lysostaphin to removed extracellular adherent bacteria. The number of bacteria internalized by endothelial cells was 9%, 25% and 21% of the total (adherent + intracellular) for wild-type 8325-4, P1 and JR80, respectively. This contrasted with the three isogenic FnBP-deficient mutants, for which no intracellular bacteria were visualised either in the standard 1mm<sup>2</sup> surface area of endothelium examined, or during detailed scanning of the monolayer in multiple fields.

It is possible that the apparent lack of internalization of the FnBP-defective mutants resulted from the low number of bacteria adherent to the monolayer rather than interruption of a specific uptake pathway. This was examined by centrifuging 10<sup>8</sup> CFU onto the monolayer at the start of the adherence assay. The numbers of internalized bacteria per mm<sup>2</sup> of endothelium were 397, 826 and 790 respectively, for 8325-4, P1 and JR80 after 60 minutes incubation at 37°C in CO<sub>2</sub>. This compared with <1 bacterium per mm<sup>2</sup> for FnBP-defective mutants of 8325-4, P1 and JR80. The lack of intracellular bacteria for all three mutants indicates that fibronectin-binding proteins are critical for internalization of *S. aureus* by endothelial cells *in vitro*.

### 3.4 Discussion.

This study has demonstrated that the *S. aureus* fibronectin-binding proteins play an important role in adhesion to live human endothelial cells. All the isogenic mutants tested that were defective in expression of fibronectin-binding proteins showed a marked reduction in adhesion to endothelial cells. This was true for two laboratory strains and a recent clinical isolate. Restoration, through plasmid complementation, of the fibronectin-binding ability of a FnBP-defective mutant also restored adherence to endothelium. Adhesion was inhibited by anti-fibronectin polyclonal and monoclonal antibodies, and by a recombinant protein based on the fibronectin-binding region of *Streptococcus dysgalactiae* FnBPB. In addition to providing evidence that staphylococcal FnBPs interact with endothelial fibronectin, these data also indicate that this is through the known interaction of the D region of FnBP with the N-terminal five type I domains of fibronectin (Sottile *et al.*, 1991). Invasion of endothelial cells was also dependent on the presence of FnBPs, an observation which is explored further during work presented in Chapter 4.

These findings are important because they cast light on a key interaction in the pathogenesis of metastatic *S. aureus* infection, the adherence of bacteria to endothelial cells. Since this organism is able to infect apparently normal bone and joint tissues, a direct interaction with endothelial cells is likely as a first step in the invasion of these deeper tissues. Fibronectin is well placed to act as a receptor in this regard. It is a normal component of the extracellular matrix on the luminal surface of an endothelial monolayer and the findings presented here are consistent with a previous report of the ultrastructural localization of fibronectin between bovine endothelial cells and adherent *S. aureus* (Vann *et al.*, 1989). Given that the defect in endothelial cell adhesion seen with the FnBP deficient mutant was restored by the presence of a multicopy plasmid encoding one of *fnbA* or *fnbB*, either FnBPA or FnBPB alone can mediate adherence of 8325-4. Understanding the relative importance of each protein in the interaction

of staphylococci with endothelial cells will require further study. The relative importance of the two FnBPs in the pathogenic potential of *S. aureus* is the subject of further study in Chapter 5.

Adhesion of *S. aureus* to live endothelial cells is rapidly followed by bacterial internalization, a process that requires the presence of bacterial fibronectin-binding proteins. Fibronectin, through its known interactions with integrin receptors present on endothelium (Albelda *et al.*, 1989), is an ideal candidate molecule to orchestrate these events. Many other invasive pathogens use integrins as cellular receptors (for review see Berendt & McCormick, 1997), and *Streptococcus pyogenes* has already been shown to invade a number of epithelial cell lines through the interaction between fibronectin and epithelial cell-surface integrins (Ozeri *et al.*, 1998). The observations of this study thus prompt the speculation that *S. aureus* invades endothelial cells through similar mechanisms. This question has been addressed during work described in Chapter 4.

Under physiological conditions, the interaction of *S. aureus* with endothelial cells takes place at greatly lower bacterial density, in whole blood and under conditions of flow. The relevant activation status of the endothelial cells *in vivo* is unknown and their phenotype will vary according to site. Important differences have been reported between microvascular and large vessel endothelium in the expression and function of host receptors for the adhesion of malaria-infected erythrocytes (McCormick *et al.*, 1997). Furthermore, for adhesion of both leukocytes and malaria-infected erythrocytes, endothelial receptors show differential adhesion under shear flow conditions, with "rolling" and "static" receptors. It therefore remains possible that under conditions of flow and cytokine activation, additional adhesion pathways operate independently of, or alongside, the fibronectin pathway. Elucidating this under the full range of conditions that might prevail *in vivo* requires further detailed studies.

Plasma proteins are also an important component of these interactions. It is surprising to find no convincing role in adhesion for fibrinogen, which has been previously reported to act as a bridging molecule (Cheung *et al.*, 1991a). There are a number of differences between the previous study and this work that may explain this, including the bacterial strains used and the method of quantifying bacterial adherence. An additional difference is that Cheung and co-workers used an assay where the endothelial cells were fixed with glutaraldehyde. This may have modified the affinity or accessibility of the binding sites on the fibronectin molecules, rendering them unable to interact with the fibronectin-binding proteins on the bacteria. This was supported by the findings of a pilot experiment performed during this thesis. Purified fibronectin immobilised to plastic was treated with glutaraldehyde in the same manner as that described by Cheung (Cheung *et al.*, 1991a). This led to a 95% and 92% reduction, respectively, in adherence of *S. aureus* 8325-4 and the clinical isolate JR80 compared with the non-treated control (data not shown). It is still possible that under different conditions of growth or activation, the fibronectin pathway plays a lesser role or that if it is inoperative, secondary adhesion mechanisms become important. Indeed, in the system used in this work, low levels of residual bacterial adherence (approximately 10-20%) were seen when the fibronectin-binding pathway was non-functional due to mutation, or the presence of rFNBD protein or anti-fibronectin antibodies. The receptor for this secondary pathway is unknown and warrants further study.

### 3.5 Chapter summary.

This study has demonstrated that the *S. aureus* fibronectin-binding proteins play an important role in adherence to and invasion of human endothelial cells *in vitro*. Isogenic mutants deficient in fibronectin-binding protein had reduced adherence to endothelial cells, while complementation of an 8325-4 FnBP-deficient mutant with a multicopy plasmid carrying an *fnb* gene restored bacterial adherence to endothelial cells. Anti-fibronectin antibodies and the recombinant form of the ligand-binding region of *Streptococcus dysgalactiae* FnBPB inhibited adherence of *S. aureus* to endothelial cells. FnBPs were also shown to be of central importance to invasion of endothelial cells. These results represent a starting point for the in-depth investigation of *S. aureus* FnBP-endothelial cell interactions.

## **Chapter 4. Fibronectin-binding protein A of *S. aureus* has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells.**

### **4.1 Chapter content.**

*S. aureus* fibronectin-binding proteins are central to the invasion of endothelium, fibronectin forming a bridge between bacterial FnBPs and host cell receptors. The aim of this study was to dissect further the mechanisms of *S. aureus* invasion of endothelial cells. This was achieved by:

- 1      Expressing a series of truncated FnBPA proteins that lacked one or more of the A, B, C or D regions on the surface of *S. aureus* and the non-invasive Gram-positive organism *L. lactis*.
- 2      Testing these strains for their adherence to fibronectin and endothelial cells, and invasion of endothelial cells *in vitro*.
- 3      Investigating the role of integrin  $\alpha_5\beta_1$  in uptake of *S. aureus* by endothelial cells.

## **4.2 Materials and Methods.**

### **4.2.1 Bacterial strains and plasmids.**

The strains and plasmids used in this study are listed in Table 4.1.

### **4.2.2 Construction of plasmids encoding truncated forms of *S. aureus* FnBPA.**

Regions of DNA encoding one or more regions of FnBPA were deleted from *fnbA* carried on plasmid pFNBA4 (Greene *et al.*, 1995; provided by Professor T. Foster) by inverse PCR, using the strategy shown in Figure 4.1 and oligonucleotide primers listed in Table 4.2. A site for the restriction enzyme *Bgl*/II was incorporated at the 5' end of each primer to facilitate ligation of the PCR product. Herculanse™ Enhanced DNA Polymerase (Stratagene) was used in accordance with the manufacturer's instructions to amplify the DNA encoding the *fnbA* gene and plasmid backbone, using an annealing temperature of 65°C. Amplified DNA was purified using a SpinX® tube (Costar®), digested with the restriction enzyme *Bgl*/II, and self-ligated with T4 DNA ligase. The plasmids constructed are listed in Table 4.1.

### **4.2.3 Verification of deletions in *fnbA*.**

Regions of *fnbA* were amplified from the plasmids (pRM1-8) described above using *Pfu* DNA Polymerase (Promega) and appropriate forward and reverse primers to generate a PCR product spanning the deletion. This was sequenced using BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), and the reactions analysed by the DNA Sequencing Facility in the Biochemistry Department, University of Oxford.



Table 4.1. Bacterial strains and plasmids

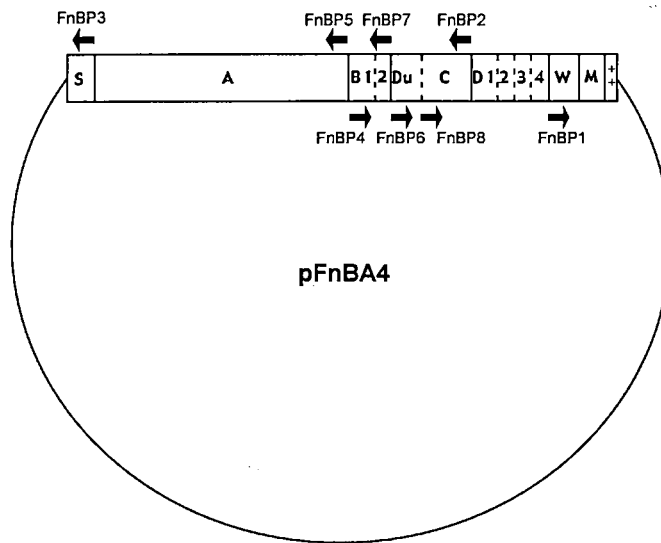
Strain/plasmid	Relevant genotype/markers	Relevant properties	Source/reference
<b>E. coli</b> DH5- $\alpha$	$\Delta$ <i>SupE44</i> $\Delta$ <i>lacU169</i> ( $\Delta$ 80) <i>lacZ</i> DM15) <i>hsdR17 recA1 ebdA1</i> <i>gyrA96 thi-1 relA1</i>	Cloning host	
<b>S. aureus</b> RN4220		Restriction-deficient 8325-4 derivative; stable maintenance of shuttle plasmids	
8325-4		FnBPA <sup>+</sup> FnBPB <sup>+</sup> ; NCTC 8325 cured of prophages	(Novick, 1967)
DU5883	<i>fnbA::Tc<sup>r</sup>fnbB::Em<sup>R</sup></i>	FnBPA <sup>-</sup> FnBPB <sup>-</sup> ; isogenic mutant of 8325-4	(Greene et al., 1995)
DU5875	<i>spa::Tc<sup>R</sup></i>	Protein A-deficient isogenic mutant of 8325-4	T.J. Foster
DU5883 (pFnBPA4)	<i>Tc<sup>R</sup>Em<sup>R</sup>Cm<sup>R</sup></i>	FnBP <sup>-</sup> strain expressing full-length FnBPA	(Greene et al., 1995)
SP1	DU5883 (pRM1)	FnBP <sup>-</sup> strain expressing FnBPA minus the A region	This work
SP2	DU5883 (pRM2)	FnBP <sup>-</sup> strain expressing FnBPA minus the B region	This work
SP3	DU5883 (pRM3)	FnBP <sup>-</sup> strain expressing FnBPA minus the Du region	This work
SP4	DU5883 (pRM4)	FnBP <sup>-</sup> strain expressing FnBPA minus the D region	This work
SP5	DU5883 (pRM5)	FnBP <sup>-</sup> strain expressing FnBPA minus the A and B regions	This work
SP6	DU5883 (pRM6)	FnBP <sup>-</sup> strain expressing FnBPA minus the B and C regions	This work
SP7	DU5883 (pRM7)	FnBP <sup>-</sup> strain expressing FnBPA minus the C and D regions	This work
SP8	DU5883 (pRM8)	FnBP <sup>-</sup> strain expressing FnBPA minus the B, C and D regions	This work
<b>L. lactis</b> MG 1363		Wild type strain which does not adhere to fibronectin or endothelial cells	Karen Schofield
SP18	MG 1363 (pKS80)Em <sup>R</sup>	Wild type strain containing expression plasmid	This work
SP9	MG 1363 (pRM9)Em <sup>R</sup>	Wild type strain expressing full length FnBPA	This work
SP10	MG 1363 (pRM10)Em <sup>R</sup>	Wild type strain expressing FnBPA minus the A region	This work
SP11	MG 1363 (pRM11)Em <sup>R</sup>	Wild type strain expressing FnBPA minus the B region	This work
SP12	MG 1363 (pRM12)Em <sup>R</sup>	Wild type strain expressing FnBPA minus the Du region	This work
SP13	MG 1363 (pRM13)Em <sup>R</sup>	Wild type strain expressing FnBPA minus the D region	This work
SP14	MG 1363 (pRM14)Em <sup>R</sup>	Wild type strain expressing FnBPA minus the A and B regions	This work
SP16	MG 1363 (pRM16)Em <sup>R</sup>	Wild type strain expressing FnBPA minus the C and D regions	This work
SP17	MG 1363 (pRM17)Em <sup>R</sup>	Wild type strain expressing FnBPA minus the B, C and D regions	This work

**Table 4.2 Oligonucleotide primers.**

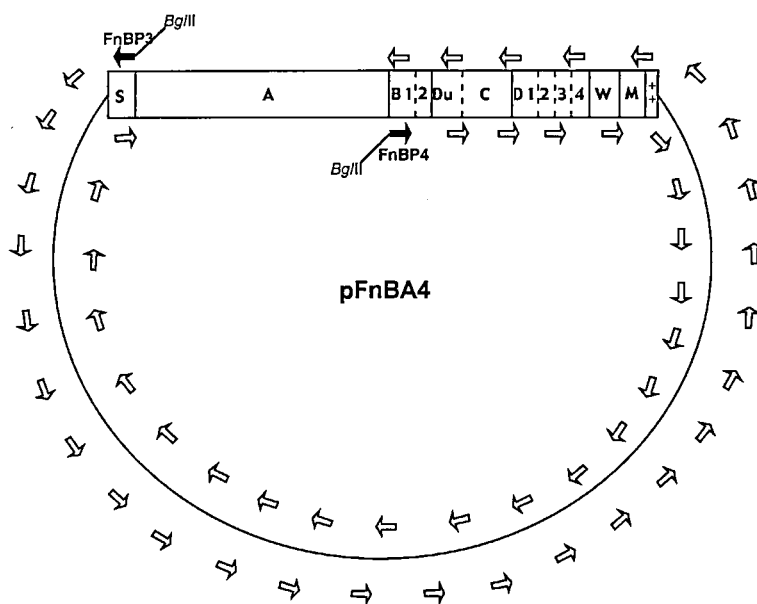
Primers	Sequence
FnBP1	5'-GAAGATCTATCGTGCCACCAACGCCAC-3'
FnBP2	5'-GAAGATCTACCTTCATAACCTAATTC-3'
FnBP3	5'-GAAGATCTTGCAGCTTCTTTGTCTTGTC-3'
FnBP4	5'-GAAGATCTGAAGAGGAATATGATTCATC-3'
FnBP5	5'-GAAGATCTAACAGTAGTTACTAAATTC-3'
FnBP6	5'-GAAGATCTTCTGAGGAATCAAATCCAATTG-3'
FnBP7	5'-GAAGATCTATTTTCATGTGTAGATTCTTC-3'
FnBP8	5'-GAAGATCTACAACAGTTGAAGATACGAAAG-3'
pKSFor	5'-GGCGGATCCGTGAAAAACAATCTTAGGTACGGC-3'
pKSrev	5'-GGCGGATCCTTATGCTTTGTGATTCTTTTATTTC-3'

Endonuclease restriction sites are underlined

**A**



**B**



**Figure 4.1 Construction of plasmids encoding *fnbA* mutants.** (A) Diagrammatic representation of the plasmid pFnBA4 illustrating the 5'-3' orientation of the binding sites of each primer (FnBP1-8) used to delete regions of the *fnbA* gene. (B) An example of the inverse PCR method used to construct deletion mutants. To delete the A region, the primers FnBP3 and 4 were used to amplify the plasmid DNA as indicated by the arrows. Restriction sites for the enzyme *Bgl*/II were incorporated at the 5' end each primer. The resulting product of this PCR amplification was digested with the enzyme *Bgl*/II and self-ligated. The restriction site for the enzyme *Bgl*/II replaces the A region.

#### **4.2.4 Confirmation of surface expression of truncated FnBPA by *S. aureus*.**

Qualitative assessment of truncated forms of FnBPA on the surface of *S. aureus* was confirmed using Western immunoblotting. Cell wall-associated proteins were extracted, separated by 7.5% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was blocked by incubation for 1 hour at room temperature in 1% BSA (v/v) in PBS, then incubated for a further hour at room temperature with anti-AB region antibodies (provided by Professor M. Höök) and anti-D region antibodies (provided by Professor M. McGavin) (1µg/ml). The membrane was rinsed and incubated in PBS containing BSA (1%, v/v) and alkaline-phosphatase conjugated protein A (Sigma) 2 µg/ml. Conjugated protein A was used to prevent non-specific binding of antibodies to protein A in the cell wall extracts.

#### **4.2.5 Cloning of *fnbA* into a *L. lactis* plasmid.**

The coding region of FnBPA was cloned downstream of a *L. lactis* constitutively expressing promoter LPS2 on the plasmid vector pKS80 (Hartford *et al.*, 2001), to give strain SP9 expressing full-length FnBPA. The genes were amplified using the plasmids pFNBA4 or pRM1-pRM8 where appropriate as templates. A single pair of oligonucleotide primers were used (pKSfor and pKSrev in Table 4.2), which incorporated a site for the restriction enzyme *Bam*HI at the 5' end. The DNA was amplified using *Pfu* DNA Polymerase (Promega) in accordance with the manufacturer's instructions. The resulting PCR product was digested with the restriction enzyme *Bam*HI and ligated into the *Bc*/I restriction site on the plasmid pKS80. The plasmids constructed are listed in Table 4.1. The ligation product was electroporated into *L. lactis* as described above.

#### **4.2.6 Confirmation of surface expression of FnBPA by *L. lactis*.**

Whole cell immunoblotting was used to confirm surface expression of full length and truncated forms of FnBPA by *L. lactis*. Bacteria were cultured

overnight in M17 broth with 0.5% glucose, 5 $\mu$ l of which was spotted onto a nitrocellulose membrane and allowed to dry at room temperature. The membrane was blocked by incubation for 1 hour at room temperature in BSA (1%, v/v) in PBS, then incubated for a 1 hour at room temperature with the antibodies used to detect FnBPA on the surface of *S. aureus*, detailed above. The membrane was rinsed in PBS and incubated in BSA containing alkaline phosphatase-labelled goat anti-rabbit antibody (0.6  $\mu$ g/ml) (Chemicon) for 1 hour. Antibody was visualised using the AP Conjugate Substrate Kit (Biorad) in accordance with the manufacturer's instructions.

#### **4.2.7 Adherence of *S. aureus* and *L. lactis* to fibronectin.**

Adherence of *S. aureus* and *L. lactis* to purified human fibronectin. 10 $\mu$ g/ml was assessed using a standardised microtitre plate assay, as described in chapter 2.

#### **4.2.8 Adherence of *S. aureus* to fibronectin in the presence of recombinant forms of FnBPA.**

Fibronectin adherence assays were performed using the microtitre plate method with the exception that recombinant forms of either full length FnBPA or subunits representing the CD (residues 584-845) (Joh *et al.*, 1994), A (residues 37-544) or B (residues 303-568) regions of *S. aureus* FnBPA were added to the wells immediately prior to bacterial inoculation at a final concentration of 0.1 $\mu$ mol (provided by Professor M. Höök). Control wells contained either recombinant truncated ClfA protein (ClfA41, residues 221-559, provided by Professor T. Foster) (O'Connell *et al.*, 1998) at a final concentration of 0.1 $\mu$ mol, or no recombinant protein. The total volume was maintained at 100 $\mu$ l in each well.

#### **4.2.9 Immunofluorescence of endothelial cell receptors.**

Confluent endothelial cells on Thermanox coverslips were dip-washed three times and placed into 24-well plates containing 500µl M199. Added to this was a mouse monoclonal antibody recognizing either integrin  $\alpha_5\beta_1$  (clone JBS5, Chemicon MAB1969), residues 383-447 of heat shock protein 60 (clone LK1, Sigma H4149); or a rabbit polyclonal antibody recognizing fibronectin (F3648, Sigma) at a dilution of 1:200. Control antibodies used were normal rabbit IgG (I5006 Sigma) or mouse IgG (M9035 Sigma). Coverslips were incubated at 37°C in CO<sub>2</sub> for 30 minutes, dip-washed, and placed into fresh 24-well plates containing FITC-labelled anti-mouse antibody (F0479, Dako) or anti-rabbit antibody (API32F, Chemicon) 1:200 in M199. After a further 30 minutes at 37°C in CO<sub>2</sub>, cells were rinsed, mounted onto glass slides and examined in a fluorescence microscope (Zeiss).

#### **4.2.10 Effect of anti-integrin and anti-Hsp60 antibodies on *S. aureus* invasion of endothelial cells.**

This was evaluated using *S. aureus* 8325-4 defective in protein A (DU5875) to avoid the confounding interaction between protein A and the Fc region of IgG. Antibodies at a final concentration of 10µg/ml were pre-incubated with endothelial monolayers for 30 minutes and remained during invasion assays. The antibodies used were the anti-Hsp60 (LK1) and the function-blocking anti-integrin  $\alpha_5\beta_1$  described above in immunofluorescence, and a control antibody to integrin  $\beta_3$  (clone B3A, MAB2023Z, Chemicon) (all were IgG1 antibodies). Assays were performed in parallel in which coverslips were pre-treated with M199 alone.

#### **4.2.11 Effect of recombinant FnBPA regions on *S. aureus* invasion of endothelial cells.**

Invasion assays were performed with the wild type *S. aureus* strain 8325-4 in the presence of 0.1 $\mu$ M of each of the following recombinant proteins: A region (residues 37-544), B region (residues 303-568), and the CD regions (residues 584-845) of *S. aureus* FnBPA (Joh *et al.*, 1994). Control wells contained either no recombinant protein or recombinant truncated ClfA protein (ClfA41, residues 221-559) (O'Connell *et al.*, 1998).

### **4.3 Results.**

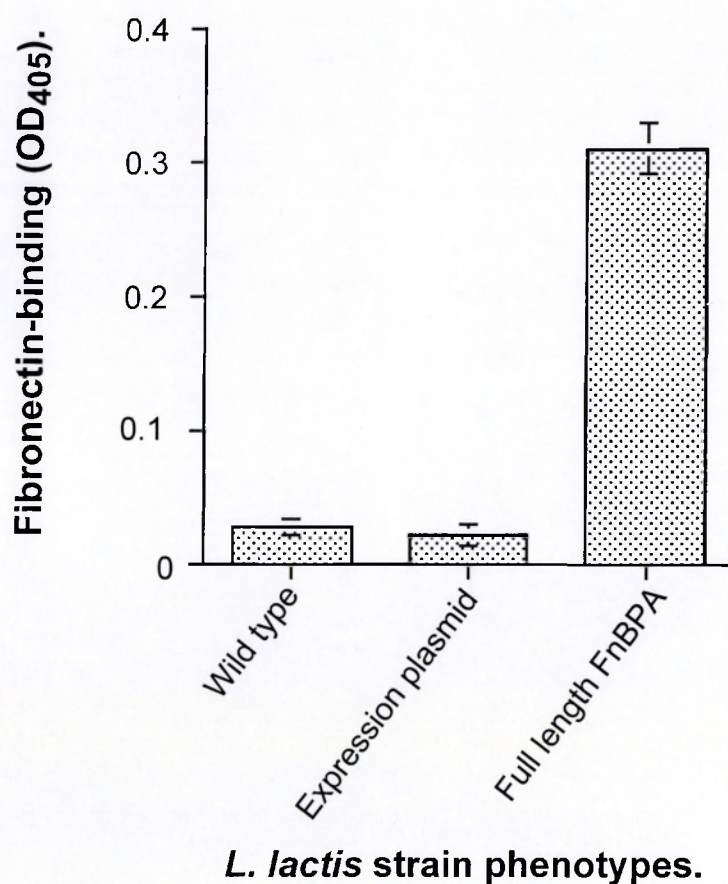
#### **4.3.1 Heterologous expression of FnBPA allows bacterial invasion of endothelial cells.**

To investigate whether *S. aureus* factors other than FnBPA are required for invasion of endothelial cells, full length FnBPA was expressed on the surface of *L. lactis*, a Gram-positive bacterium that does not adhere to fibronectin or endothelium. Functional expression of FnBPA by this strain was demonstrated using the microtitre plate fibronectin adherence assay (Fig. 4.2). The expression of full length FnBPA by *L. lactis* conferred the ability to invade endothelial cells ( $P < 0.001$ ) (Fig. 4.3). This indicates that a *S. aureus* co-receptor is not required for FnBPA-mediated invasion of endothelial cells.

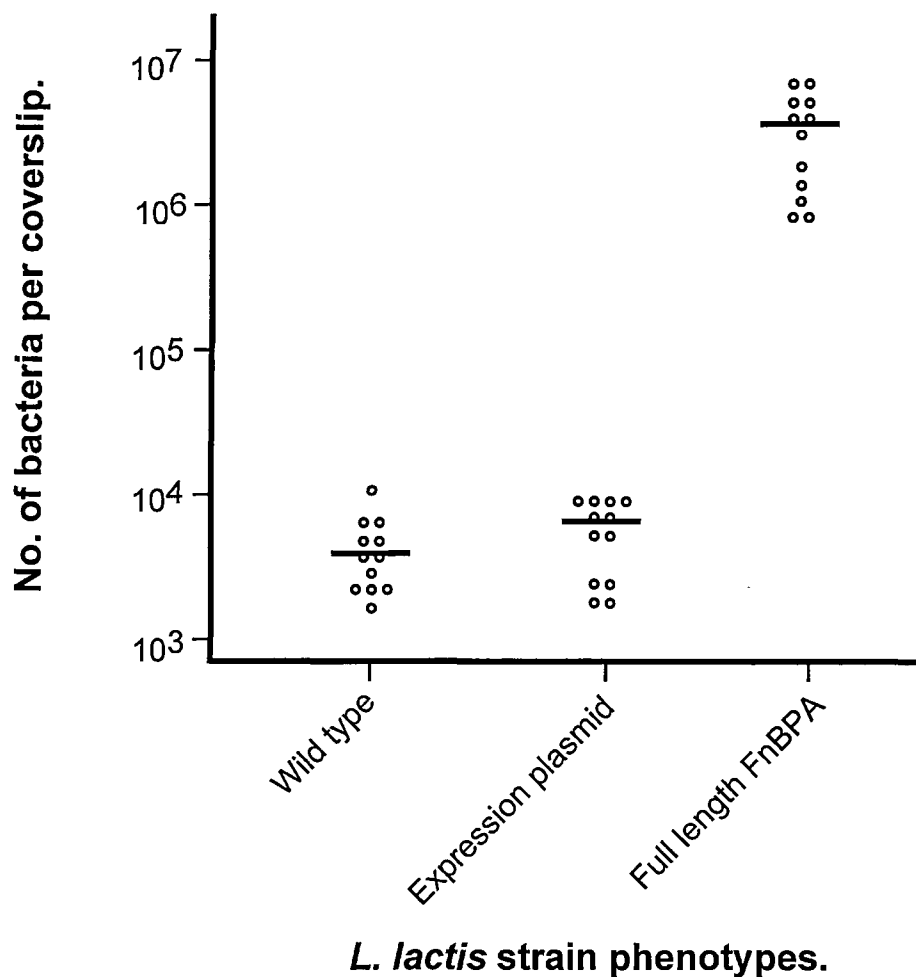
#### **4.3.2 FnBPA has multiple, substituting fibronectin-binding regions that mediate adherence to fibronectin and attachment and invasion of endothelial cells.**

To investigate which regions of FnBPA are involved in invasion of endothelial cells, truncated FnBPA proteins lacking one or more of the A, B, Du or D regions were expressed on the surface of *S. aureus* and *L. lactis*. The Du region was deleted rather than the entire C region as it was previously considered to be the ligand binding site of the C region. Figure 4.4 shows a diagrammatic representation of each mutant form of FnBPA constructed.

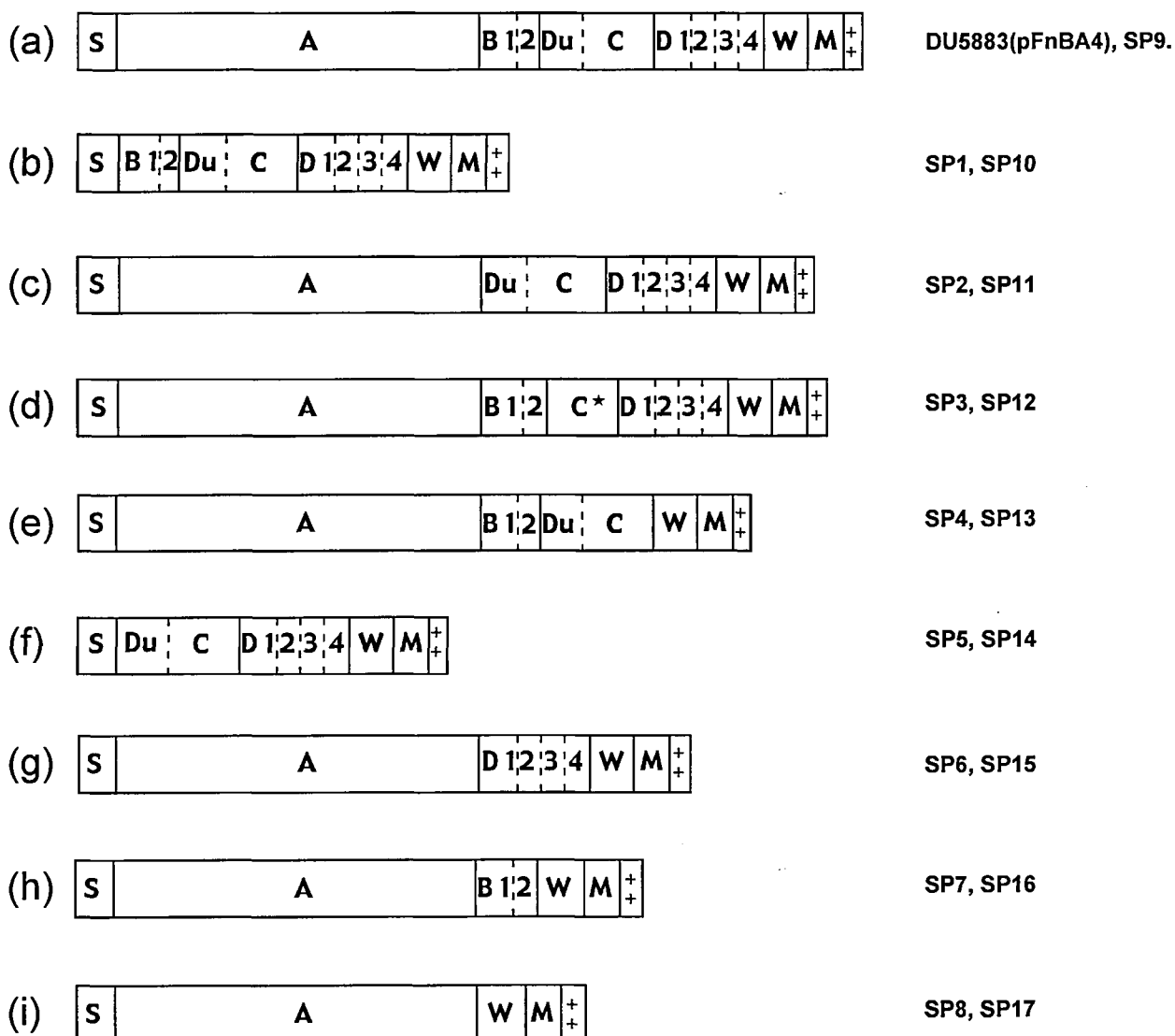




**Figure 4.2 Functional expression of FnBPA on the surface of *L. lactis* confers the ability to adhere to fibronectin.** Wild type *L. lactis* (MG1363); *L. lactis* carrying the expression plasmid pKS80; *L. lactis* expressing full length FnBPA. Results are shown as the means  $\pm$  SEM.

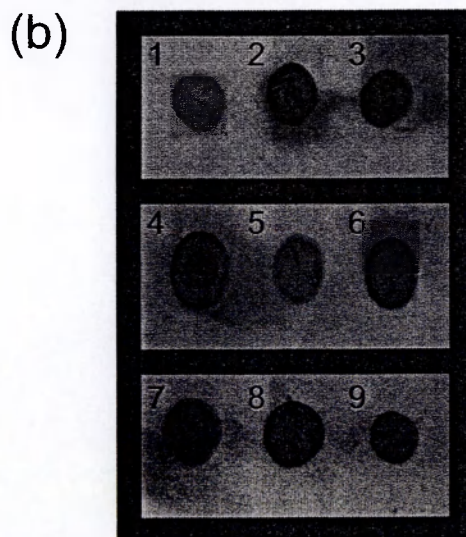
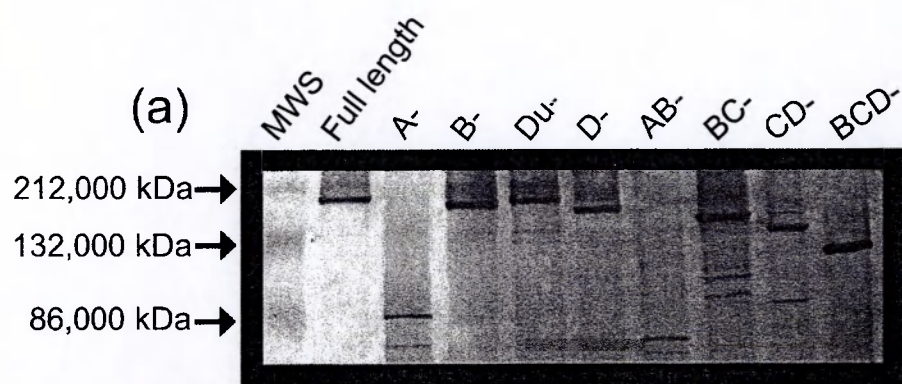


**Figure 4.3 Functional expression of FnBPA on the surface of *L. lactis* confers the ability to invade endothelial cells.** Wild type *L. lactis* (MG1363); *L. lactis* carrying the expression plasmid pKS80; *L. lactis* expressing full length FnBPA. The results represent the counts for two independent experiments, and the thick bar represents the median value.



**Figure 4.4 Fibronectin-binding protein A constructs.** Diagrammatic designations, the first is for the construct expressed on the surface of *S. aureus*, the second on the surface of *L. lactis*. (S: signal sequence; A: region with fibrinogen-binding activity; B: region containing two repeats; C: region containing the Du repeat which has fibronectin-binding activity; D: fibronectin-binding region with representation of constructs lacking regions of FnBPA. (a) full length; (b) A<sup>-</sup>; (c) B<sup>-</sup>; (d) Du<sup>-</sup>; (e) D<sup>-</sup>; (f) AB<sup>-</sup>; (g) BC<sup>-</sup>; (h) CD<sup>-</sup>; (i) BCD<sup>-</sup>. Each construct has two four repeats (D1-D4); W: wall-spanning region; M: membrane-spanning region). The star indicates that the Du repeat has been deleted from the C region.

Surface expression of truncated proteins by *S. aureus* was verified by Western immunoblotting of cell wall proteins using anti-FnBPA antibodies (Fig. 4.5a). This confirmed that all truncated proteins had been expressed on the bacterial cell surface. Full length fibronectin-binding proteins have an apparent molecular mass of approximately 180 kDa on SDS-PAGE gel; due to their acidic nature this is higher than predicted from the amino acid sequence. Relative decreases in apparent molecular mass were observed for the truncated proteins (Fig. 4.5a). It was also observed that loss of the A region had a destabilizing effect on the integrity of FnBPA which can be visualized as degradation in the A<sup>-</sup> and the AB<sup>-</sup> strains (Fig. 4.5a). Surface expression of all truncates expressed by *L. lactis* was confirmed by whole cell immunoblotting (Fig. 4.5b).



**Figure 4.5 Verification of surface-expression of FnBPA constructs by *S. aureus* and *L. lactis*.** (a) Visualisation of fibronectin-binding protein A constructs by Western immunoblotting of cell wall-associated protein extracts from *S. aureus*. Lane 1, molecular weight standards, Lane 2: full length FnBPA (strain DU5883 (pFnBPA4)); Lane 3-10: FnBPA lacking the following regions: A<sup>-</sup>; B<sup>-</sup>; Du<sup>-</sup>; D<sup>-</sup>; AB<sup>-</sup>; BC<sup>-</sup>; CD<sup>-</sup>; BCD<sup>-</sup>. (b) Wholecell immunoblot verifying surface expression of the FnBPA constructs on the surface of *L. lactis*. 1: wild type *L. lactis* containing expression plasmid; 2: full length FnBPA; 3-9: FnBPA lacking the following regions: A<sup>-</sup>; B<sup>-</sup>; Du<sup>-</sup>; D<sup>-</sup>; AB<sup>-</sup>; CD<sup>-</sup>; BCD<sup>-</sup>.

Adherence to immobilized fibronectin was evaluated for all *S. aureus* and *L. lactis* mutants (Fig. 4.6). Deletion of regions individually and in pairs had no effect on the ability to adhere to fibronectin. Loss of adherence was only seen for *S. aureus* or *L. lactis* strains expressing a truncated protein consisting of the A region alone ( $P < 0.001$  in both cases). Adherence to fibronectin of *S. aureus* or *L. lactis* strains expressing truncated proteins consisting of the A and B regions indicates the presence of fibronectin-binding activity within the B region. The expression of a given FnBPA truncate appeared to confer similar fibronectin-binding ability to *S. aureus* and *L. lactis*.

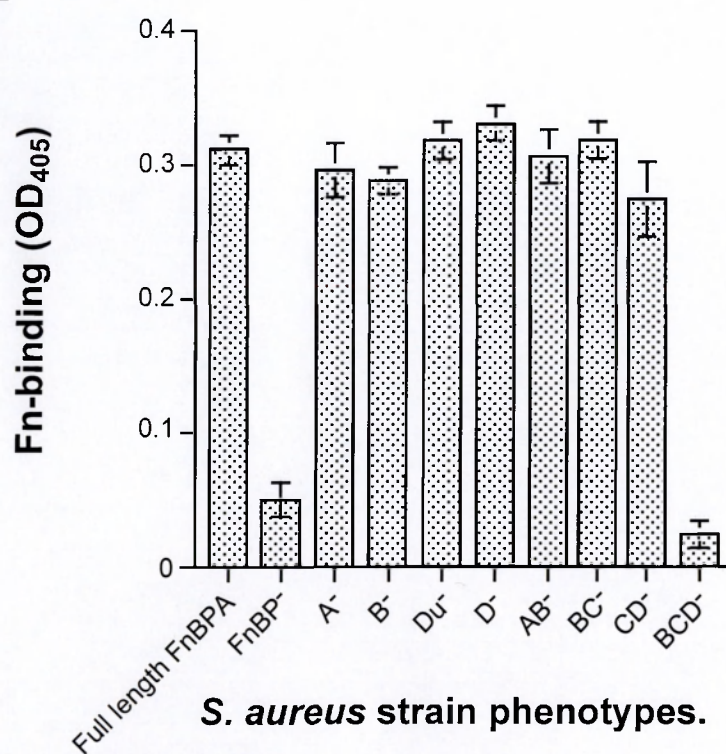
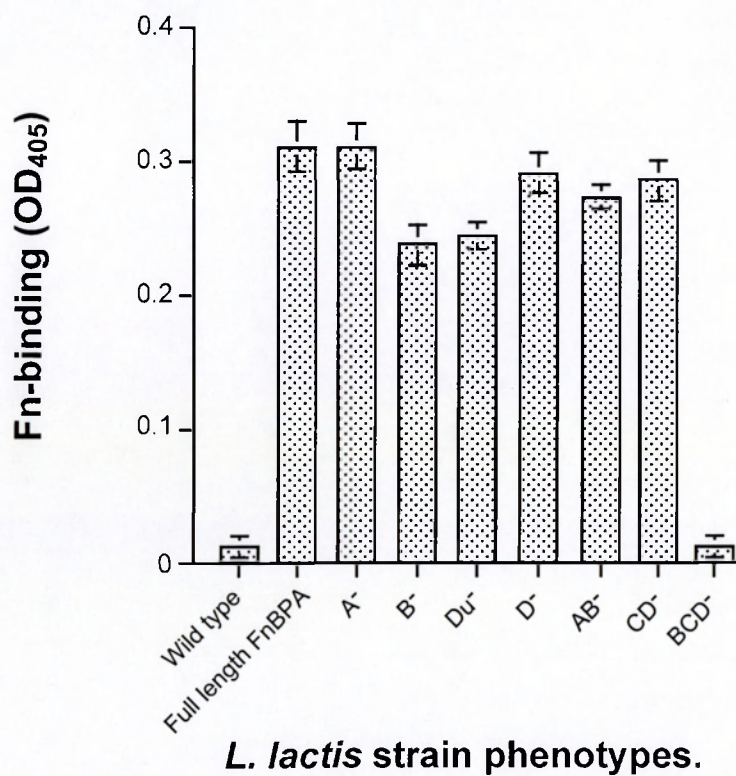
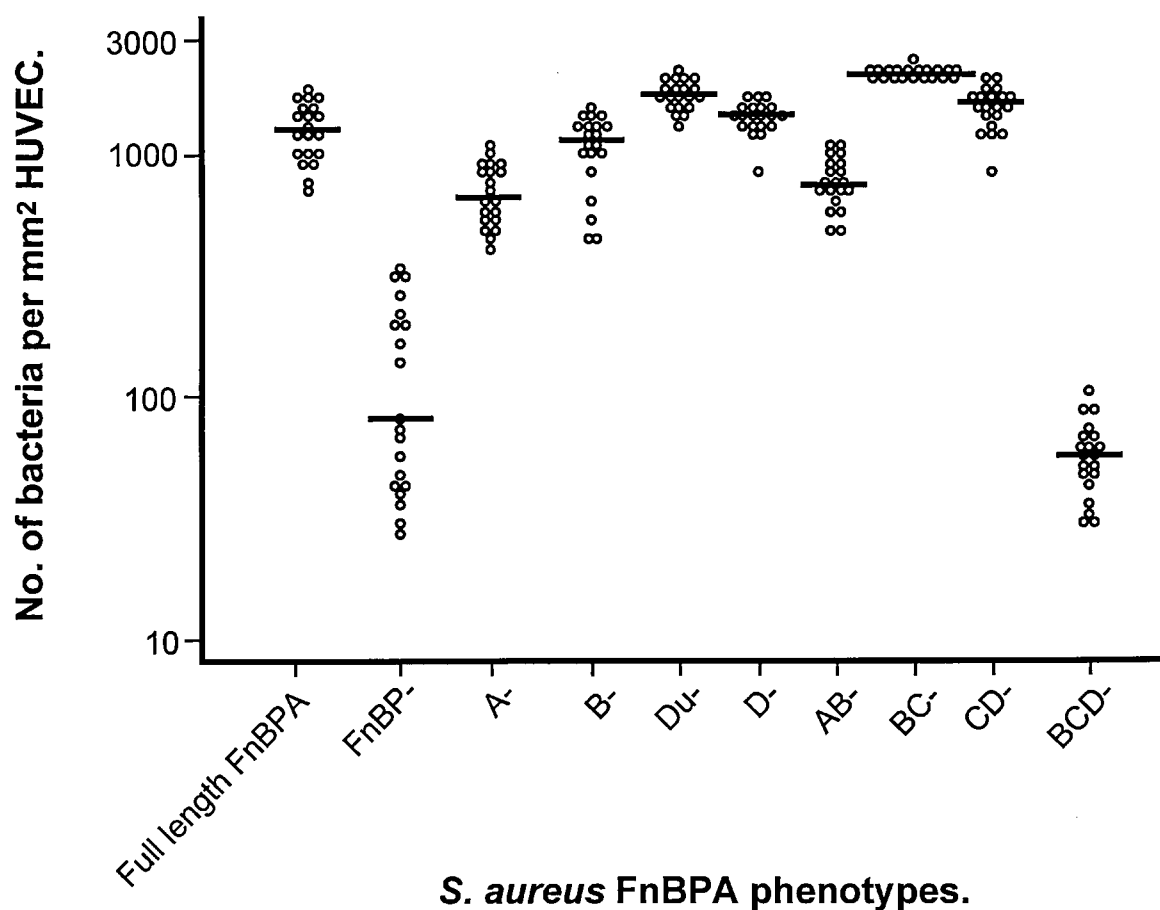
**A****B**

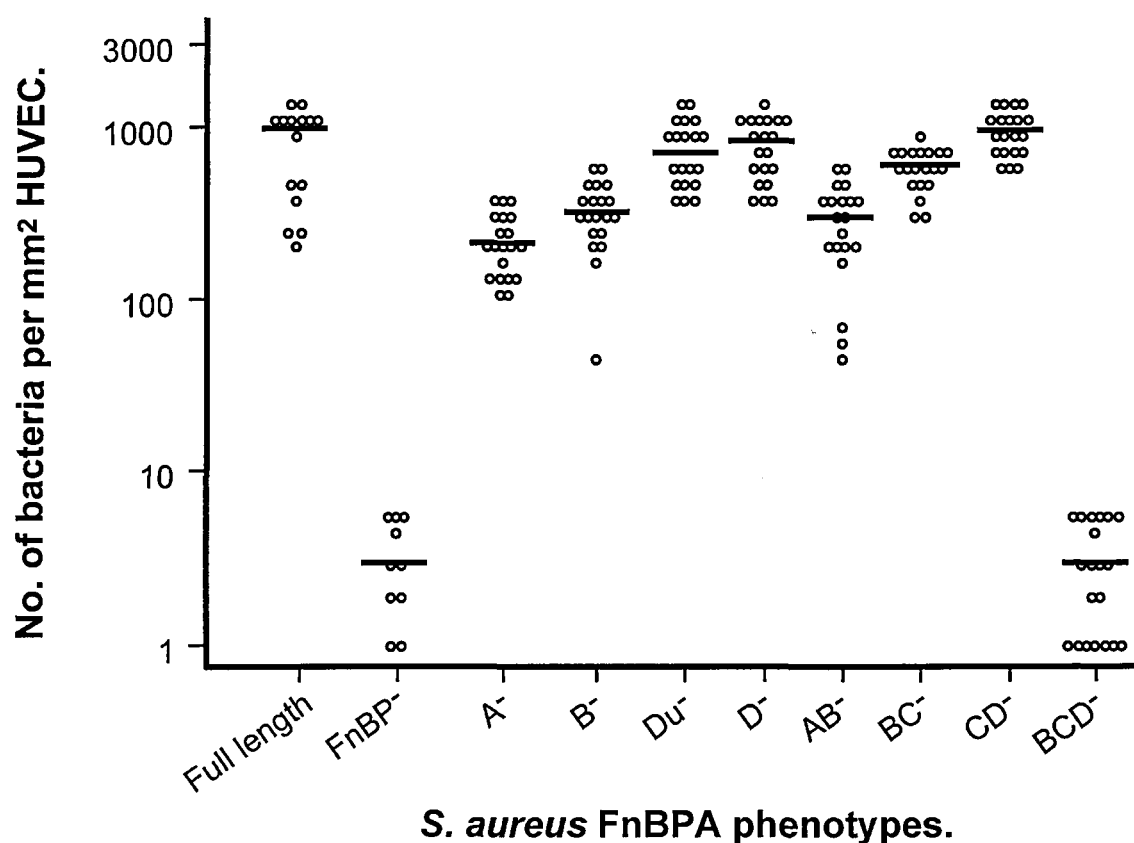
Figure 4.6 Adherence to fibronectin of bacterial strains expressing FnBPA constructs. (a) *S. aureus*, and (b) *L. lactis* expressing full length and truncated forms of FnBPA. Results are shown as the means  $\pm$  SEM.

To determine the role of each region of FnBPA in the interaction with endothelial cells, assays were performed with the *S. aureus* FnBPA mutants described above. The initial adherence event and the subsequent uptake process were examined by parallel assays of attachment and invasion combined, and invasion alone (Fig. 4.7 and 4.8). Invasion was demonstrated for strains expressing FnBPA truncates that lacked one or two of the B, C and D regions. Invasion assays were performed for all *L. lactis* FnBPA mutants (Fig. 4.9). These behaved in a similar manner to the *S. aureus* FnBPA mutants, emphasizing the lack of secondary *S. aureus* factors required for invasion of endothelial cells. Invasion of *S. aureus* and *L. lactis* was not lost until all fibronectin-binding activity was abolished (i.e. when the B, C and D regions were simultaneously deleted) ( $P < 0.001$ ), illustrating that adherence to fibronectin is the crucial factor in endothelial cell invasion. Comparison of the results for *S. aureus* invasion with those for *S. aureus* adhesion plus invasion demonstrated that bacterial counts for mutants in the two assays were directly proportional. It was observed that strains defective in fibronectin binding (strains DU5883 and SP8) adhered to HUVEC at a low level (Fig. 4.7), suggesting that *S. aureus* may have a second adhesin for endothelium.

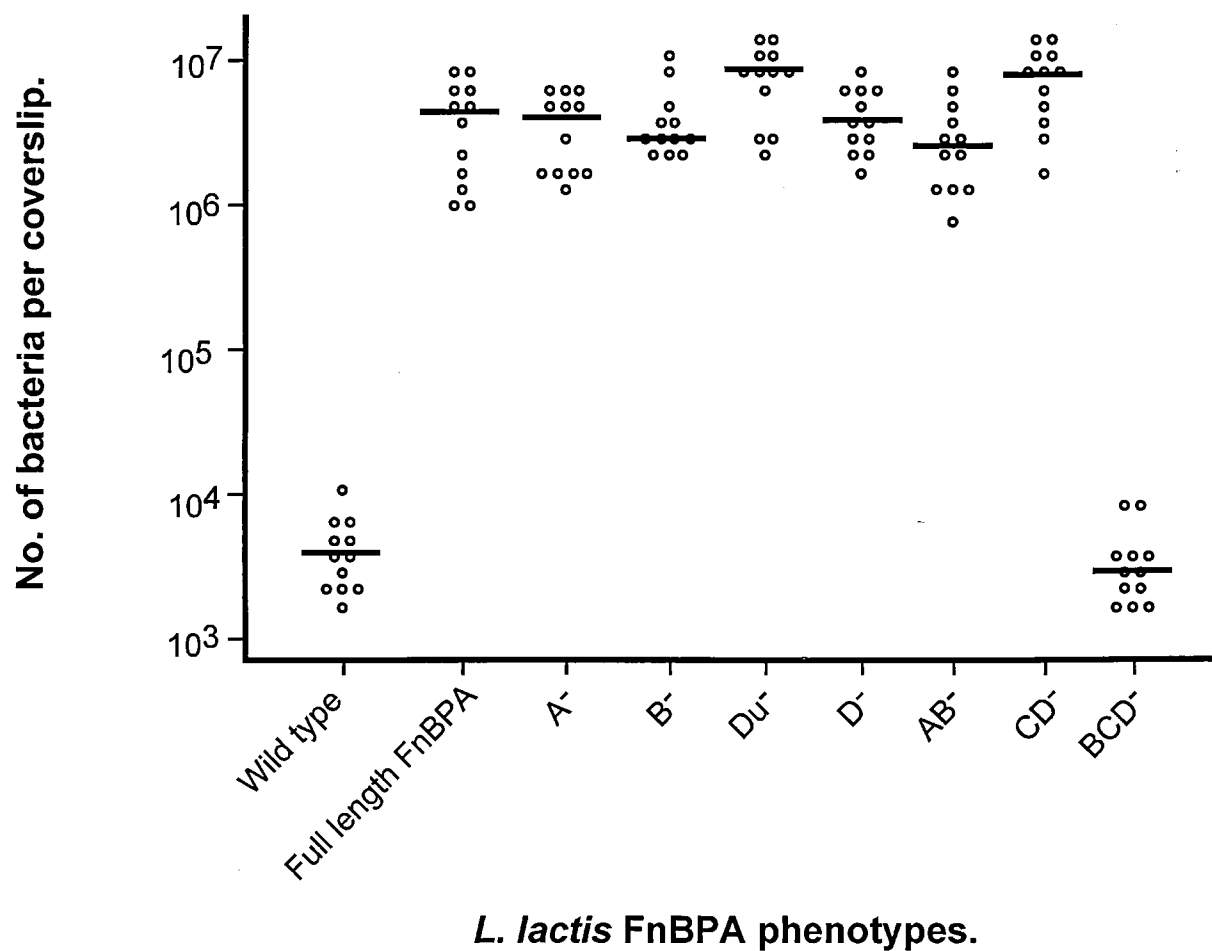




**Figure 4.7 Adherence to endothelial cells by *S. aureus* strains expressing FnBPA constructs.** The number of associated bacteria (adherent and intracellular) per mm<sup>2</sup> of endothelium for strains expressing the following FnBPA constructs: full length FnBPA; FnBP<sup>-</sup>; A<sup>-</sup>; B<sup>-</sup>; Du<sup>-</sup>; D<sup>-</sup>; AB<sup>-</sup>; BC<sup>-</sup>; CD<sup>-</sup>; BCD<sup>-</sup>. The open dots represent 20 counts per strain and the thick bars represent the median value.



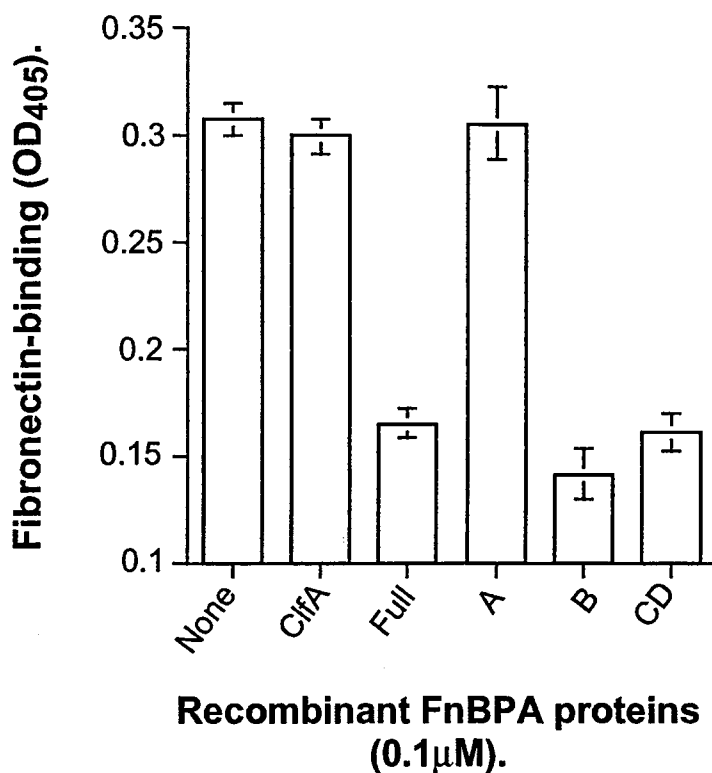
**Figure 4.8 Invasion of endothelial cells by *S. aureus* strains expressing FnBPA constructs.** The number of internalized bacteria per mm<sup>2</sup> of endothelium for strains expressing the following FnBPA constructs: full length FnBPA; FnBP<sup>-</sup>; A<sup>-</sup>; B<sup>-</sup>; Du<sup>-</sup>; D<sup>-</sup>; AB<sup>-</sup>; BC<sup>-</sup>; CD<sup>-</sup>; BCD<sup>-</sup>. The open dots represent 20 counts per strain and the thick bars represent the median value.



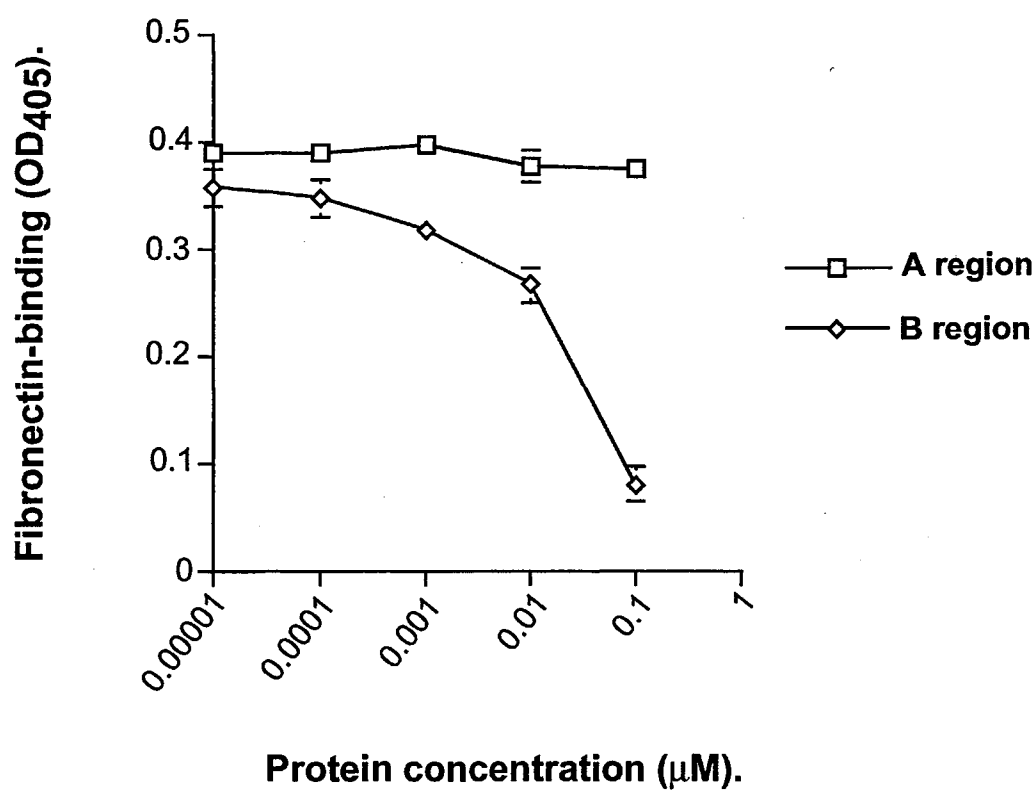
**Figure 4.9 Invasion of endothelial cells by *L. lactis* strains expressing FnBPA constructs.** The number of internalized bacteria per coverslip of endothelium for *L. lactis* strains expressing the following FnBPA constructs: wild type; full length FnBPA; A<sup>-</sup>; B<sup>-</sup>; Du<sup>-</sup>; D<sup>-</sup>; AB<sup>-</sup>; CD<sup>-</sup>; BCD<sup>-</sup>. The open dots represent colony counts for each strain and the thick bars represent the median value.

#### **4.3.3 Recombinant B, C and D regions of FnBPA can competitively inhibit *S. aureus* adherence to fibronectin.**

It has been demonstrated previously that the D regions of *S. aureus* FnBPA and *Streptococcus dysgalactiae* FnBPB can competitively inhibit *S. aureus* adherence to fibronectin (chapter 3; Sinha *et al.*, 1999). To verify that the B region has fibronectin-binding activity, the effect of recombinant protein incorporating this region was examined on bacterial adherence to fibronectin. Microtitre plate adherence assays were performed in the presence of the recombinant forms of full length FnBPA, A, B or CD regions. Recombinant B region protein was found to be unstable, but could be stabilized by the inclusion of 314 amino acids of the C terminal A region (personal communication, Professor M. Höök). For clarity, this protein is subsequently referred to as recombinant B region protein. Recombinant full length FnBPA, the B protein and the CD protein reduced adherence of *S. aureus* to fibronectin ( $P < 0.001$ ), while the control protein (recombinant truncated fibrinogen-binding protein, ClfA41, residues 221-559) had no effect ( $P > 0.05$ ) (Fig. 4.10). Recombinant A region protein had no blocking effect ( $P > 0.05$ ). A gradient of recombinant protein concentrations was used to further verify the competitive nature of blocking by the B region relative to the A region. Figure 4.11 illustrates the increase in blocking of *S. aureus* adherence to fibronectin as the concentration of B protein increases.



**Figure 4.10 Inhibition of *S. aureus* adherence to fibronectin by recombinant FnBPA proteins.** Adherence of wild type *S. aureus* 8325-4 to fibronectin in the presence of equimolar amounts of recombinant proteins of FnBPA or the fibrinogen-binding protein ClfA. None: no exogenous protein added; ClfA: recombinant fibrinogen-binding protein (residues 221-559); Full: recombinant full length FnBPA; A: recombinant A region; B: recombinant B region; CD: recombinant CD regions. The results are shown as the means  $\pm$  SEM.



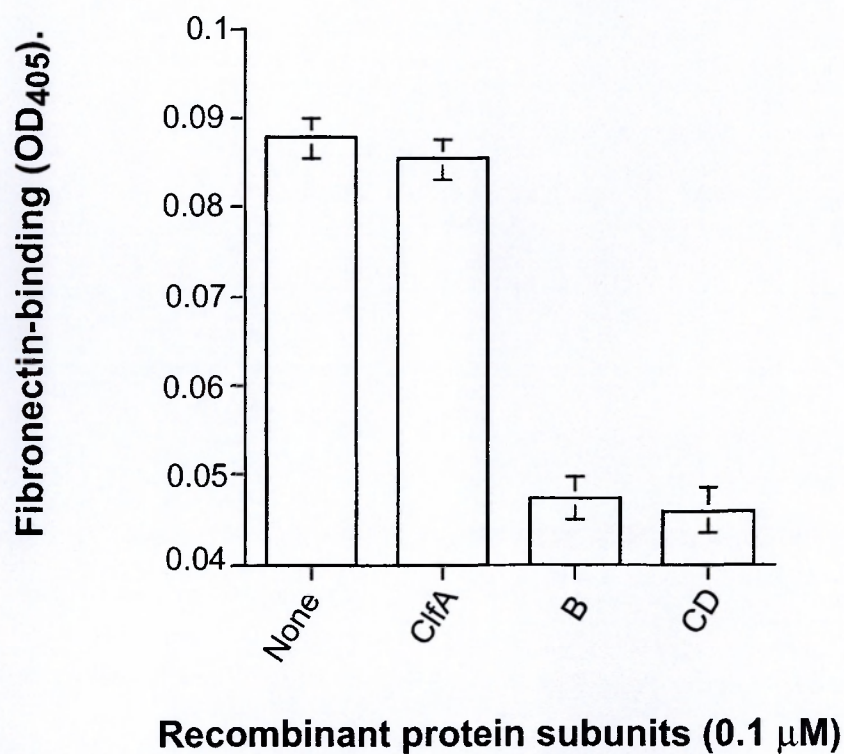
**Figure 4.11 Inhibition of *S. aureus* adherence to fibronectin by recombinant FnBPA proteins.** Adherence of wild type *S. aureus* 8325-4 to fibronectin in the presence of increasing amounts of recombinant A and B proteins of FnBPA. Results are shown as the means  $\pm$  SEM.

#### **4.3.4 Adherence of *S. aureus* to fibronectin via the B region can be blocked by recombinant CD region protein.**

Fibronectin adherence assays were performed using the *S. aureus* strain expressing the A and B regions of FnBPA only (thus adherence is via the B region), in the presence of recombinant B or CD protein. As anticipated, the B recombinant protein blocked adherence to fibronectin ( $P > 0.001$ ). The presence of CD protein also led to an equally efficient inhibition of adherence ( $P > 0.001$ ) (Fig. 4.12). This suggests that the B region interacts with fibronectin at the same site as the C and D regions, or that the interaction with an alternative fibronectin-binding site is blocked through steric hindrance.

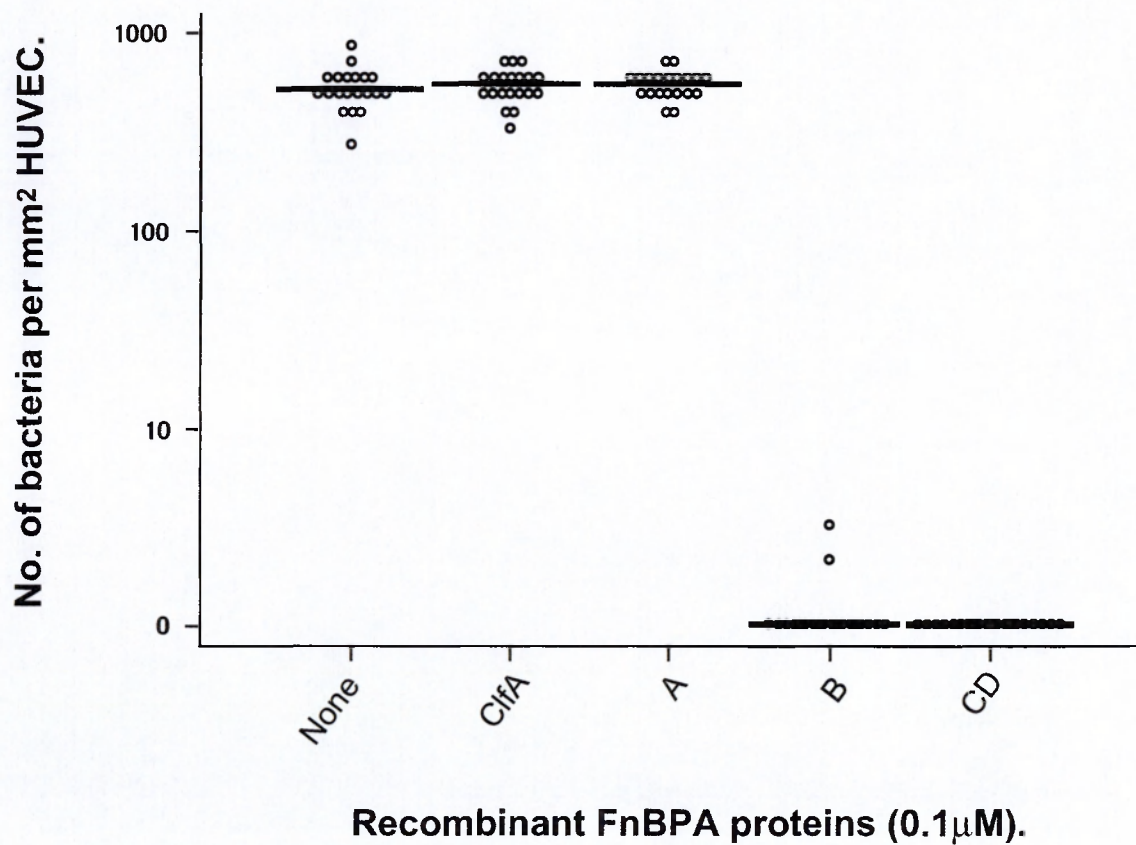
#### **4.3.5 Recombinant B, C and D regions of FnBPA can competitively inhibit *S. aureus* invasion of endothelial cells.**

*S. aureus* invasion assays were performed in the presence of recombinant forms of FnBPA. Invasion was blocked in the presence of the recombinant B region and CD regions of FnBPA ( $P < 0.001$ ), while the control protein ClfA (as described above) and the A region of FnBPA had no blocking effect ( $P > 0.05$ ) (Fig. 4.13).



**Figure 4.12 Adherence of *S. aureus* to fibronectin via the B region is blocked by recombinant CD region protein.** Adherence to fibronectin of the *S. aureus* strain which expresses only the A and B regions of FnBPA in the presence of equimolar amounts of recombinant B or CD region proteins. None: no exogenous protein added; ClfA: recombinant fibrinogen-binding protein; Full: recombinant full length FnBPA; B: recombinant B regions; CD: recombinant CD regions. Results are shown as the means  $\pm$  SEM.



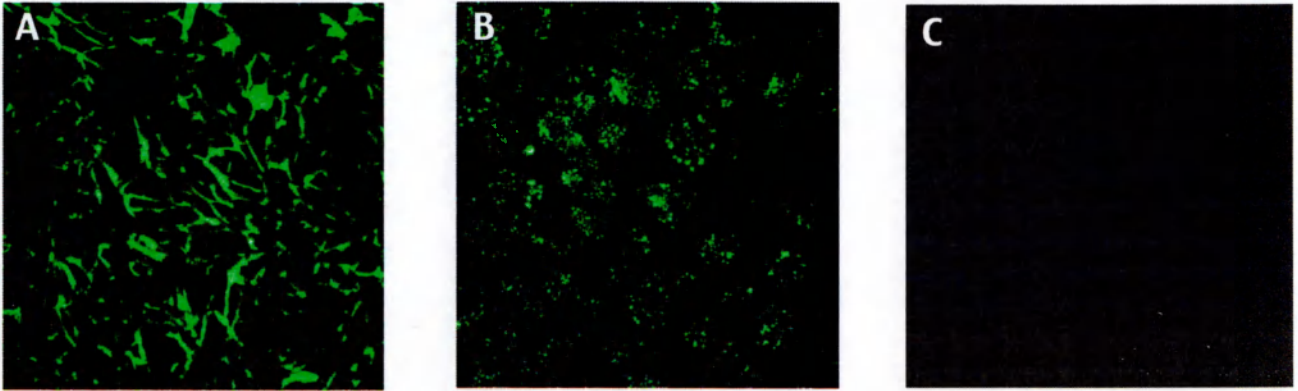


**Figure 4.13 Invasion of endothelium by *S. aureus* in the presence of recombinant FnBPA proteins.** Invasion of endothelium by wild-type *S. aureus* 8325-4 in the presence of equimolar amounts of recombinant FnBPA proteins. None: no recombinant protein added; ClfA: recombinant fibrinogen-binding protein; A: recombinant A region; B: recombinant B region; CD: recombinant CD regions. Results are shown as the means  $\pm$  SEM.

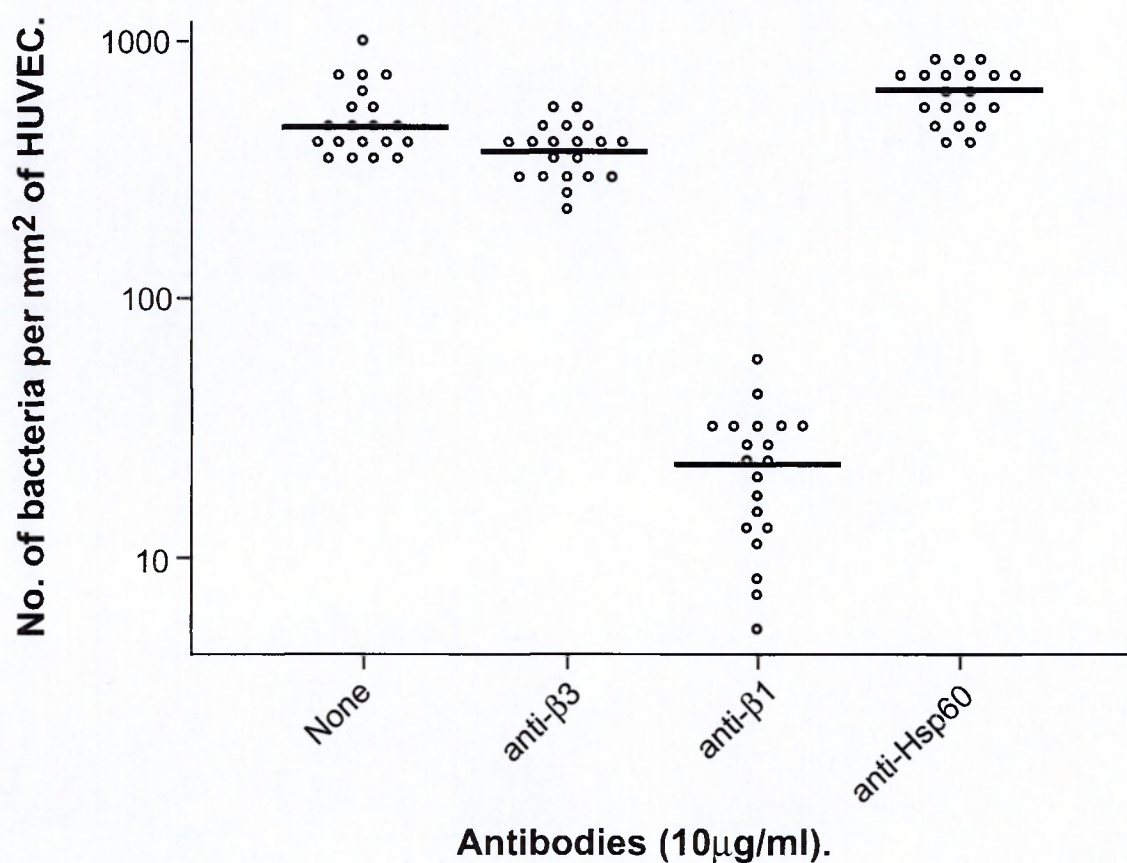
#### 4.3.6 *S. aureus* invasion of endothelial cells is mediated by the integrin $\alpha_5\beta_1$ .

Immunofluorescent microscopy was used to confirm the presence of fibronectin and integrin  $\alpha_5\beta_1$  on the surface of the endothelial monolayer (Fig. 4.14). To test the role of integrin  $\alpha_5\beta_1$  in invasion of endothelial cells, *S. aureus* invasion assays were performed after pre-incubation of endothelium with function-blocking anti- $\alpha_5\beta_1$  antibodies (Fig. 4.15). An antibody to the  $\alpha_3$  integrin subunit was used as the control. Invasion by *S. aureus* was reduced in the presence of the anti- $\alpha_5\beta_1$  antibody ( $P < 0.001$ ), while the control antibody had no effect ( $P > 0.05$ ).

It has been reported that *S. aureus* fibronectin-binding protein interacts with Hsp60 on the membrane of bovine mammary epithelial cells, and that the anti-Hsp60 antibody, LK1, reduced bacterial internalization (Dziewanowska *et al.*, 2000). The relevance of these findings to *S. aureus* invasion of endothelial cells was examined. Immunofluorescence failed to demonstrate recognition by LK1 of surface-expressed epitopes of Hsp60 on human endothelial cells. In addition, pre-treatment of the monolayer with LK1 had no effect on invasion of endothelium by *S. aureus* ( $P > 0.05$ ) (Fig. 4.15).



**Figure 4.14 Immunofluorescent microscopy of endothelial cells.** Endothelial monolayers labelled with antibodies recognising either: (a) fibronectin, (b) integrin  $\alpha_5\beta_1$ , or (c), Hsp60 (LK1, recognizing residues 383-447). Magnification X40.



**Figure 4.15 Integrin  $\alpha_5\beta_1$  mediates *S. aureus* invasion of endothelial cells.** Invasions assays were performed after pre-incubation of endothelium with 10μg/ml of the following antibodies: none: no antibody; anti- $\alpha_3\beta_1$  antibody (control); anti- $\alpha_5\beta_1$  antibody; and anti-Hsp60 antibody (LK1). The open dots represent the 20 counts per antibody and the thick bar shows the median value.

#### 4.4 Discussion.

The expression of fibronectin-binding proteins is common to many diverse species of pathogenic bacteria including *Borrelia burgdorferi* (Probert *et al.*, 2001), *Listeria monocytogenes* (Gilot *et al.*, 1999), and *Mycobacteria tuberculosis* (Armitige *et al.*, 2000). However, a role in host cell invasion has only been ascribed for the fibronectin-binding protein of staphylococcal (Peacock *et al.*, 1999) and streptococcal (Molinari *et al.*, 1997) species. *S. aureus* expresses many adhesins that bind host proteins, but to date the fibronectin-binding protein is the only endothelial cell invasin to be identified (Peacock *et al.*, 1999). Others have confirmed the role of FnBPs as invasins for endothelial cells (Sinha *et al.*, 1999), and for other cell types including bovine mammary gland cells (Lammers *et al.*, 1999), epithelial cells (Dziewanowska *et al.*, 2000; Sinha *et al.*, 1999), fibroblasts (Fowler *et al.*, 2000) and osteoblasts (Ahmed *et al.*, 2001). In a number of non-endothelial cell types, invasion has been shown to be dependent on the integrin  $\alpha_5\beta_1$  (Dziewanowska *et al.*, 2000; Fowler *et al.*, 2000; Sinha *et al.*, 1999). Unresolved questions before this study was conducted included the role of accessory bacterial or host molecules in the adhesion and uptake process, the structure-function relationship of the *S. aureus* fibronectin-binding proteins and the role of  $\beta_1$  integrins in endothelial cell invasion.

This work has shown that expression of FnBPA is not only necessary, but is also sufficient to mediate invasion of endothelial cells by *S. aureus*. This is consistent with the findings of others using transformed human embryonic kidney cells (Sinha *et al.*, 2000). Heterologous expression of functional FnBPA in the non-adhesive Gram-positive organism *L. lactis* conferred the ability to invade endothelial cells, extending previous observations that deletion of FnBPs from *S. aureus* was associated with a poorly adhesive, non-invasive phenotype (Peacock *et al.*, 1999). Having established that FnBPA is solely responsible for invasion of endothelial cells, the contribution made by each region of this protein was then examined. *S. aureus* expressing FnBPA constructs deficient in one or more of the

four regions termed A, B, C and D were initially tested for adherence to immobilised fibronectin. The D repeat region of FnBPA was previously considered to be the main fibronectin-binding region. It seemed reasonable, therefore, to predict that deletion of the D region would result in a reduction in adherence to fibronectin, and that simultaneous deletion of the C and D regions would lead to loss of function as a fibronectin-binding protein. However, adherence to fibronectin of *S. aureus* expressing FnBPA constructs deficient in either one or both of these binding regions was maintained. The existence of a third fibronectin-binding region within the B region was demonstrated by the failure of *S. aureus* expressing FnBPA deficient in the B, C and D regions to adhere to fibronectin. This concurs with findings that a recombinant FnBPA protein consisting of the A and B regions interacts with fibronectin (Miyamoto *et al.*, 2001).

Having defined the fibronectin-binding ability of *S. aureus* expressing FnBPA constructs deficient in one or more region, the interaction of these strains with endothelial cells was examined. Invasion of endothelial cells by *S. aureus* strains expressing FnBPA constructs defective in one region (C or the A, B or C region) or two regions (AC, BC or CD) was demonstrated. The only strains that were unable to invade endothelial cells were those devoid of FnBPs or expressing FnBPA deficient in the B, C and D regions. The presence of recombinant B region protein in the invasion assay inhibited adhesion, verifying that this region also interacts with fibronectin on the surface of endothelium. This is consistent with a previous report that a recombinant AB region protein reduced invasion of a mouse fibroblast line by *S. aureus* (Fowler *et al.*, 2000). The observation here that the soluble CD regions exhibit cross-inhibition suggests that although FnBPA has multiple binding regions, they either all interact with a similar region of fibronectin or that steric hindrance is occurring.

Lack of endothelial cell invasion by FnBP mutants of *S. aureus* could be due to either lack of initial adherence, or failure of uptake once bound to the

endothelial surface. In response to this, parallel assays were performed to assess either invasion alone, or adherent extracellular plus internalized bacteria combined. Although strains of *S. aureus* defective in FnBPs or expressing the A domain alone were not internalized, adherence of these strains was not completely abolished. Thus, it is possible to conclude that FnBPs are essential for invasion, but that *S. aureus* has a second adhesin for endothelial cells. This is consistent with findings of a previous study in which 21% of an inoculum of *S. aureus* 8325-4 defective in FnBPs adhered to MAC-T cells compared with 35% for the isogenic parent (Dziewanowska *et al.*, 1999). The A region did not appear to interact with endothelium, the results for both invasion and adhesion/invasion reflecting those for the FnBP defective mutant.

Study of the host cell determinants involved in uptake of *S. aureus* by endothelium reported here was preceded by reports from others that invasion of 293 cells, Hep-2 cells and mouse fibroblasts required the integrin  $\alpha_5\beta_1$ . However, the function blocking anti- $\alpha_5\beta_1$  antibody failed to block uptake by MAC-T cells in a study that implicated Hsp60 as the mediator of invasion (Dziewanowska *et al.*, 2000). This work therefore focused on the effect of function-blocking antibodies to integrin  $\alpha_5\beta_1$ , and the anti-Hsp60 antibody LK1 which blocked invasion of MAC-T cells. A significant reduction was demonstrated in invasion in the presence of anti-integrin  $\alpha_5\beta_1$  antibody, but there was no reduction in invasion in the presence of anti-Hsp60 LK1 antibody. In eukaryotic cells Hsp60 is mainly localized in the mitochondria where it functions as a molecular chaperone involved in protein folding, but smaller amounts are present at other sites including the plasma membrane (Soltys & Gupta, 1996; Soltys & Gupta, 1997). Expression of Hsp60 does not occur in rat endothelial cells *in vitro* unless stressed, for example following pre-incubation with cytokines (Xu *et al.*, 1994). In addition, antibody LK1, recognizing residues 383-447 of Hsp60, does not recognize surface expressed epitopes of Hsp60 on rat endothelial cells (Xu *et al.*, 1994). A role for Hsp60 in adherence of *S. aureus* to stressed endothelial cells

has not been excluded, but based on current evidence it is unlikely to be a major host factor in the invasion of resting cells. Although further work is required using antibodies that recognize Hsp60 epitopes expressed by stimulated human endothelium, there is a difficult but nonetheless fundamental issue that needs to be addressed to put this and other host receptor work into context. That is, what is the initial level of endothelial cell receptor expression *in vivo* when these cells become associated with *S. aureus* during invasive human disease?

This study has investigated the mechanism of invasion of resting human endothelial cells by *S. aureus*. The model is one in which adherence to endothelium occurs through the interaction between bacterial fibronectin-binding proteins and fibronectin, followed by uptake mediated by integrin  $\alpha_5\beta_1$ . The presence of fibronectin on the endothelial cell surface was demonstrated by immunofluorescence and soluble fibronectin was not added to the adherence assays, so it can be concluded that *S. aureus* interacts with host cell-associated fibronectin in this system.

It has also been demonstrated that FnBPA has multiple, substituting fibronectin-binding regions including the B region. Why should a protein have this degree of apparent redundancy? One explanation is that the presence of multiple binding regions allows other regions to substitute activity should one become neutralized by host antibodies. Alternatively, multiple binding sites may allow bacteria to adhere despite negative effects found *in vivo* such as circulatory flow.

Future investigations should include a detailed analysis of the role of plasma proteins, the effect of flow and endothelial stimulation, and the interplay between these factors. Some of this work is underway or has been reported by other investigators. The effect of flow has been examined by two groups; Reddy *et al.* found that shear stress prevented FnBP-mediated adherence of *S. aureus* 8325-4 to resting endothelial cells (Reddy & Ross, 2001). Shenkman *et al.* did not find a difference in adherence of wild type between static and flow



conditions, although adherence of mutants defective in *sar* or *sar/agr* was reduced by around 50% (Shenkman *et al.*, 2001). Pre-treatment of endothelial cells with human  $\alpha$ -thrombin increased bacterial adherence under conditions of flow (Shenkman *et al.*, 2000). The presence of fibrinogen in the bacterial suspension also increased adherence, although detection of binding was performed using radiolabelled bacteria, and the possibility of adherence of agglutinated bacteria was not excluded (Shenkman *et al.*, 2000).

#### **4.5 Chapter summary.**

This study has dissected the FnBPA-dependent mechanism of invasion of human umbilical vein endothelial cells by *S. aureus*. FnBPA was shown to have multiple, substituting fibronectin-binding regions, each capable of conferring both adherence to fibronectin and endothelial cells, and endothelial cell invasion. Invasion of endothelial cells did not require additional *S. aureus* factors, and as with other cell types was mediated by integrin  $\alpha_5\beta_1$ .

## **Chapter 5. Clinical isolates of *S. aureus* exhibit diversity in *fnb* genes and adhesion to human fibronectin.**

### **5.1 Chapter content.**

*S. aureus* FnBPs have been implicated in the pathogenesis of prosthetic material-related infection (Greene *et al.*, 1995; Vaudaux *et al.*, 1984; Vaudaux *et al.*, 1993), and chapters 3 and 4 of this thesis have described their involvement in the interaction with human endothelial cells *in vitro*. Despite the likely importance of these adhesins in disease pathogenesis, FnBPs expressed by clinical isolates have not been characterized extensively. This chapter examines the phenotypic and genotypic characteristics of the FnBPs of a large population of recent clinical isolates of *S. aureus* associated with nasal carriage or disease. This was achieved by:

- 1      Examining adherence of 163 clinical isolates of *S. aureus* associated with carriage and disease to immobilised fibronectin, and comparing adherence with clinical association.
  
- 2      Studying further a subgroup of isolates with poor adherence to fibronectin using:
  - i.      Western ligand affinity blotting.
  - ii.     Adherence assays following growth in the presence of a protease inhibitor, thereby examining whether poor adhesion could be explained by proteolysis of adhesins during growth.
  
- 3      Determining the number of genes encoding fibronectin-binding proteins using Southern hybridization and dot blot, and examining this in relation to fibronectin adherence, and to source of the clinical isolate.

## **5.2 Materials and methods.**

### **5.2.1 Bacterial isolates.**

*Staphylococcus epidermidis* NCTC 11047 was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, U.K. The 163 clinical isolates of *S. aureus* evaluated were cultured from specimens submitted to the microbiology laboratories of the John Radcliffe Hospital, Oxford, UK (N=103), and the Auckland, Green Lane and Middlemore Hospitals, Auckland, NZ (N=60), between 1993 and 1996. All were methicillin-susceptible by the comparative disc diffusion method.

### **5.2.2 Classification of isolates according to clinical history.**

Isolates were divided into four groups according to the clinical history of the patient from whom they were isolated: nasal carriage in the absence of *S. aureus* disease (n=44); native-valve endocarditis (n=34); primary septic arthritis and/or osteomyelitis (n=56); and orthopaedic implant-associated infection (n=29). The first three categories represented isolates from individuals with community-acquired carriage or infection. Endocarditis isolates were from patients who satisfied published criteria for definite endocarditis (Durack *et al.*, 1994). Isolates from patients with implant-associated infection were cultured from operative samples, the presence of infection being confirmed by the clinical appearance of infection, histology of intra-operative tissue biopsies and the isolation of *S. aureus* from multiple independent samples.

### **5.2.3 Bacterial adherence assay.**

Adherence of bacterial isolates to purified human fibronectin 10µg/ml was assessed using a microtitre plate assay. Each isolate was tested in quadruplicate in an individual assay and each experiment was performed three times. All assay plates included *S. aureus* 8325-4 as a positive control, and PBS without bacteria

as a negative control. The OD<sub>405</sub> used in the analysis was the mean value for a given strain, minus the background OD<sub>405</sub> taken from the reading for the negative control on the same plate.

#### **5.2.4 Determining the number of *fnb* genes in *S. aureus* isolates using Southern hybridization and DNA dot blot.**

DNA fragments used as *fnb* probes were generated by PCR amplification. Using sequences in the GenBank database, oligonucleotide primers were designed to amplify unique gene fragments encoding region A of *S. aureus* 8325-4 FnBPA and FnBPB. The primer sequences 5'-CAACAACCAGCAAATATAG-3' (forward) and 5'-CTGTGTGGTAATCAATGTC-3' (reverse) were used to amplify a probe corresponding to bases 431-1792 of *fnbA* (as assigned by the GenBank sequence); and 5'-TAGAAACTTCGCGAGTTG-3' (forward) and 5'-TCAAGTTCATAGGAGTAC-3' (reverse) to amplify a probe corresponding to bases 830-1997 of *fnbB*. Template DNA was plasmid pFNBA3 or pFNBB3 which carry *S. aureus* 8325-4 *fnbA* and *fnbB*, respectively as described by Greene *et al.* 1995. The magnesium concentration used was 1.5 mM, and amplification consisted of 30 cycles of 94°C for 1 min, 50°C for 1 and 72°C for 1 min 30sec. Aliquots were analyzed by 1% agarose gel electrophoresis, purified using a DNA extraction kit and DIG-dUTP labelled by PCR using the method recommended by the suppliers (Boehringer Mannheim). DNA hybridization was otherwise performed by the method of Southern (Southern, 1992).

#### **5.2.5 Statistical analysis.**

This was performed using the Statview 4.5 statistical software package (Abacus, Berkeley, LA), and the Stata statistical software package (Stata Corp., College Station, Texas). Categorical variables were compared using Fisher's

exact test. Normally distributed variables were compared between groups using the unpaired t-test, and skewed data using the Kruskal-Wallis test.

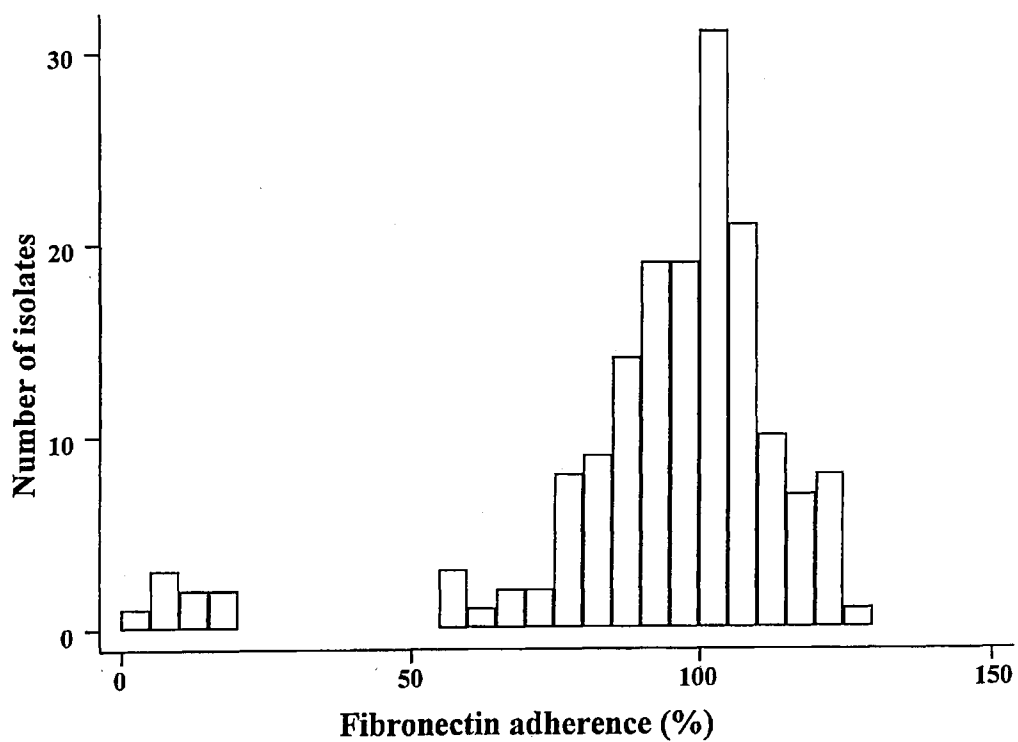
### 5.3 Results.

#### 5.3.1 Clinical isolates of *S. aureus* adhere variably to fibronectin and include poor binders.

Adherence of 163 clinical isolates was variable, as shown in Figure 5.1. Isolates clustered into two groups, the adherence of 155 isolates (95.1%) ranging from 56%-124.7% of the mean adherence for *S. aureus* 8325-4, while the adherence of the remaining 8 isolates (4.9%) was less than 20% (range 4.2% to 19.6%).

The isolates were divided into the clinical categories as defined above to determine whether adherence to fibronectin (as a percentage of *S. aureus* 8325-4 adherence), was comparable between the four groups. Adherence of the bacterial group associated with orthopaedic implant-associated infection was significantly greater than that for the remaining groups (Table 5.1). This finding was reproduced on re-analysis of the data set after exclusion of the 8 isolates that adhered poorly.

The prevalence of isolates with poor adherence to fibronectin was: nasal colonising, 0/44 (0%); endocarditis, 2/34 (6%); septic arthritis/osteomyelitis, 5/56 (9%); and orthopaedic implant-associated, 1/29 (3%). There was no significant difference in the proportion of low binders between the groups ( $p=0.196$ , Fisher's exact test).



**Figure 5.1 Adherence of 163 clinical isolates of *S. aureus* to fibronectin.** Fibronectin adherence is expressed as a percentage of the mean of *S. aureus* 8325-4.

**Table 5.1 Comparison between adherence of *S. aureus* to fibronectin and clinical association.**

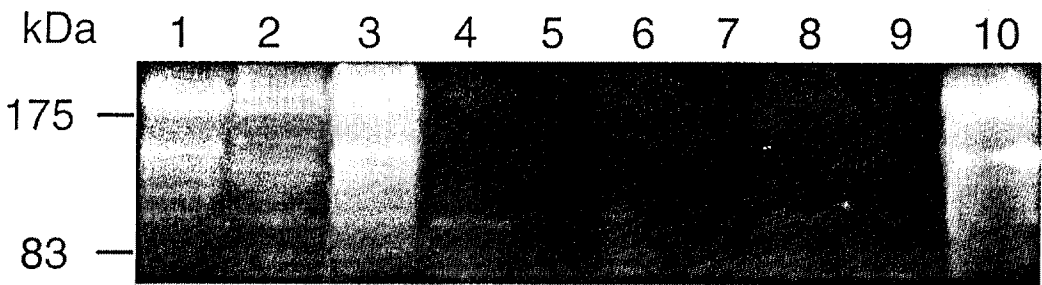
	Nasal carriage	Endocarditis	Septic arthritis/osteomyelitis	Orthopaedic implant-associated
Median adherence, % of <i>S. aureus</i> 8325-4	97.95	94.6	98.1	107.7
Range	56.0-124.7	4.2-128.2	6.9-121.9	19.6-124.4
Interquartile range (25%-75%)	86.8-103.25	84.1-103.9	87.5-103.5	102.0-113.8
Comparison of adherence between groups:	p value:	p value:	p value:	p value:
Orthopaedic implant-associated	0.0004	0.0004	0.0001	-
Septic arthritis/osteomyelitis	0.74	0.79	-	-
Endocarditis	0.61	-	-	-

Comparison of adherence between the four groups was carried out using the Kruskal-Wallis test. Adjustments have not been made for multiple comparisons.



**5.3.2 Two of 8 poor fibronectin-binding isolates have detectable FnBPs on ligand affinity blotting.**

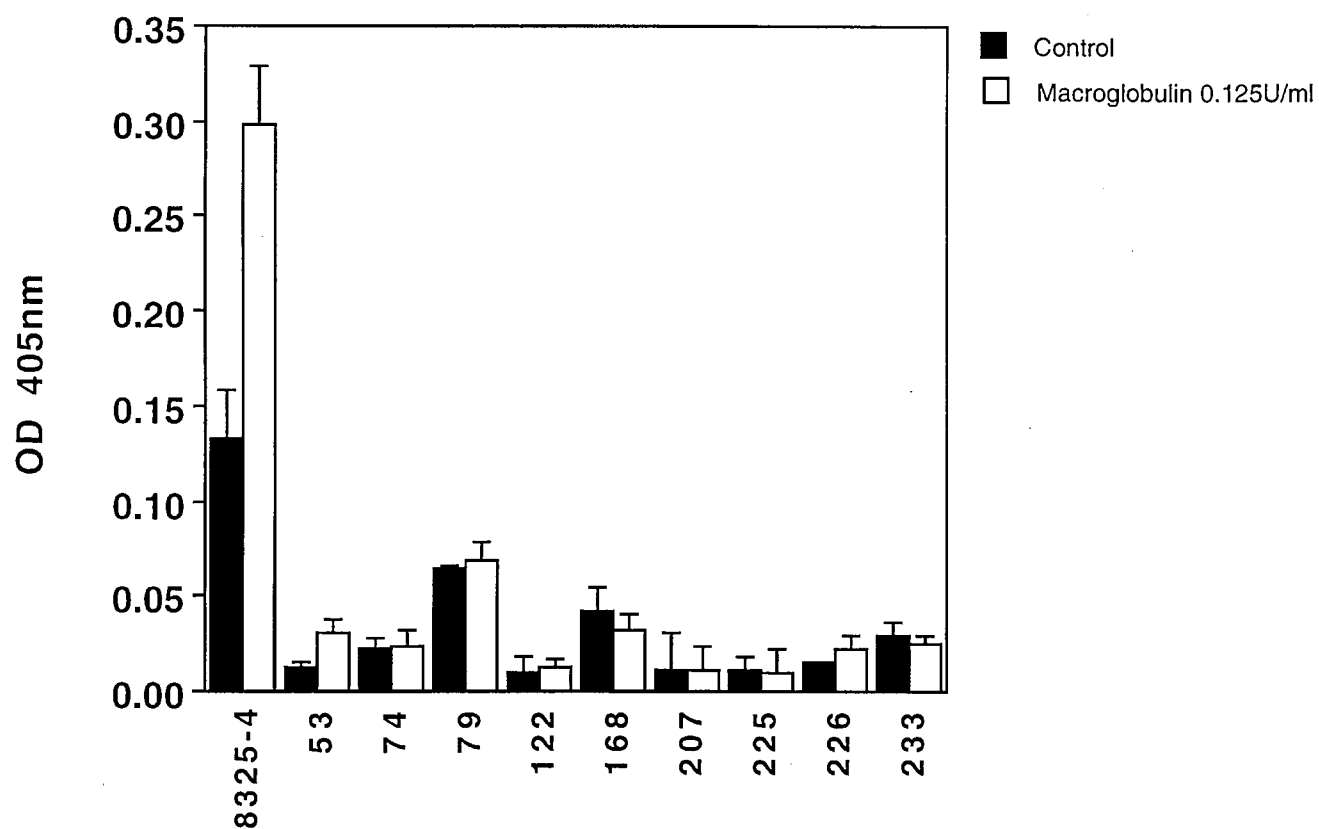
The presence of functional FnBPs was evaluated by Western ligand affinity blotting for the 8 poor binders and a clinical isolate that had equivalent adherence to *S. aureus* 8325-4 (isolate number 80 from a patient with endocarditis), together with *S. aureus* 8325-4 as the positive control (Fig. 5.2). A band with an apparent molecular mass of approximately 180kDa was present in *S. aureus* 8325-4, 80, and two out of 8 poor binders (isolates numbered 53 and 233, lanes 3 and 10 in Fig. 5.2, respectively). The adherence of 53 and 233 to fibronectin was 19.6% and 9.7% respectively, compared with *S. aureus* 8325-4.



**Figure 5.2 Visualisation of fibronectin-binding proteins by Western ligand affinity blotting of cell wall-associated protein extracts.** Lane 1, *S. aureus* 8325-4; lane 2, isolate number 80 whose adherence to fibronectin was comparable to 8325-4; lanes 3 to 10, isolates with poor adherence to fibronectin by microtitre assay. These are numbered consecutively as follows: isolate 53, 74, 122, 168, 207, 225, 226 and 233.

### **5.3.3 Poor adherence to fibronectin does not result from cleavage of FnBPs by bacterial proteases.**

Modification of the adhesive phenotype and cell surface protein profile of the FnBPs of *S. aureus* by endogenous V8 protease has been described, whereby FnBPs undergo proteolysis during growth in broth culture (McGavin *et al.*, 1997). The possibility that poor adherence to fibronectin was related to cleavage of FnBP by bacterial proteases secreted during culture was evaluated for the 8 poor binders. Isolates were incubated for 18 hours in TSB at 37°C in air under constant rotation, either in the presence or absence of the universal protease inhibitor  $\alpha_2$ -macroglobulin 0.125U/ml (Boehringer Mannheim). The positive and negative controls (for the presence or absence of an effect of  $\alpha_2$ -macroglobulin on adherence to fibronectin) were *S. aureus* 8325-4 and *Staphylococcus schleiferi* NCTC 12218, respectively. The fibronectin adherence assay was performed using the microtitre plate assay. The OD<sub>405</sub> for the 8 clinical isolates and the negative control were not significantly different between the treated and untreated samples ( $p > 0.05$  for all isolates, paired t-test), while that for *S. aureus* 8325-4 increased by 114% following culture in the presence of  $\alpha_2$ -macroglobulin (Fig. 5.3).



**Figure 5.3** Effect of bacterial proteases on adherence of *S. aureus* to fibronectin.

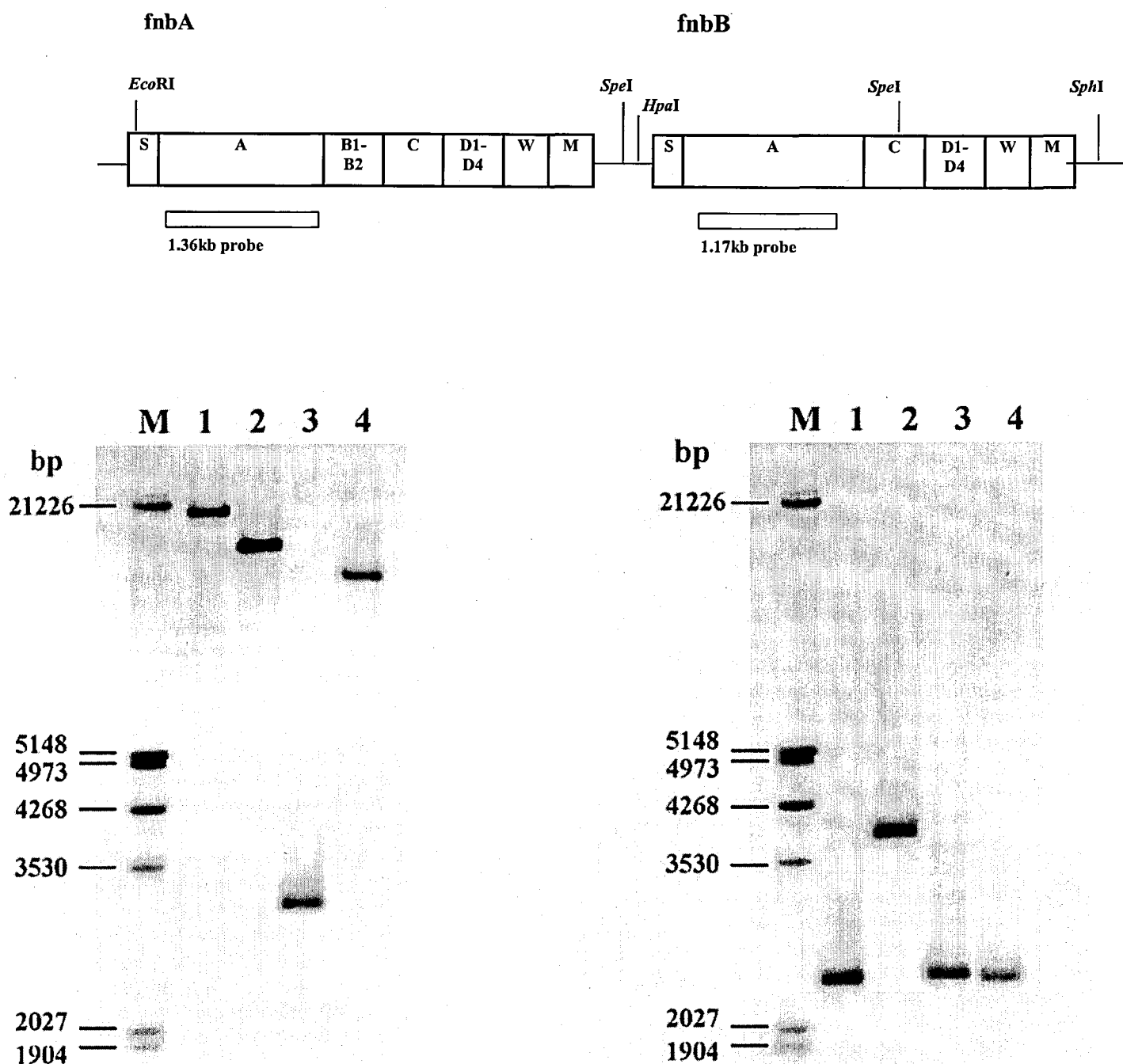
### 5.3.4 Clinical isolates vary in their complement of *fnb* genes.

The specificity of the two probes for region A of *fnbA* or *fnbB* was confirmed by Southern hybridization of genomic DNA from *S. aureus* 8325-4, as follows. DNA was cleaved with several different enzymes or combinations of enzymes and probed with the *fnbA*- or *fnbB*-specific probes. In each case the A probe hybridized to a fragment of different size compared to the B probe (Fig. 5.4).

Southern dot blot was used to evaluate the number of *fnb* genes in the 163 clinical isolates. *S. aureus* 8325-4 and *S. epidermidis* NCTC 11047 were used as positive and negative controls, respectively. Independent dot blots were performed on two occasions. Overall, 126 of 163 isolates had two detectable genes (77%), and 37 of 163 had one detectable gene (23%), (Table 5.2).

**Table 5.2 Number of *fnb* genes detected by Southern dot blot in 163 clinical isolates of *S. aureus*.**

Clinical association	<i>fnb</i> genes detected by Southern dot blot, number (%)			
	<i>fnbA</i> <sup>+</sup> <i>fnbB</i> <sup>+</sup>	<i>fnbA</i> <sup>+</sup> <i>fnbB</i> <sup>-</sup>	<i>fnbA</i> <sup>-</sup> <i>fnbB</i> <sup>+</sup>	<i>fnbA</i> <sup>-</sup> <i>fnbB</i> <sup>-</sup>
Nasal carriage	28 (63.6)	16 (36.4)	0	0
Endocarditis	29 (85.3)	5 (14.7)	0	0
Septic arthritis/osteomyelitis	47 (83.9)	9 (16.1)	0	0
Orthopaedic implant-associated	22 (75.9)	6 (20.7)	1 (3.4)	0
Total (no., % of all isolates)	126 (77.3)	36 (22.1)	1 (0.6)	0



**Figure 5.4 Southern hybridization analysis of the *fnb* loci of *S. aureus* 8325-4.**

Top diagram shows map of the *fnb* locus of 8325-4 to demonstrate the *fnbA* and *fnbB* probes and position of the restriction sites used. Chromosomal DNA was cleaved with *SpeI*, lane 1; *HpaI*, lane 2; *EcoRI/SpeI*, lane 3; and *SphI/SpeI*, lane 4, and probed with: (i) a 1.36kb probe specific for the *fnbA* gene of 8325-4 (left gel), or (ii) a 1.17kb probe specific for the *fnbB* gene of 8325-4 (right). Lane M, molecular size marker.

### **5.3.5 There is a relationship between the number of *fnb* genes and invasive disease.**

The number of isolates with two *fnb* genes were as follows: nasal carriage 28/44, (63.6%); endocarditis 29/34, (85.3%); septic arthritis/osteomyelitis 47/56 (83.9%); and orthopaedic implant-associated 22/29 (75.5%), ( $p = 0.07$ , 4 x 2 Fisher's exact). The number of isolates with two genes in the community-acquired invasive disease groups (endocarditis + septic arthritis and/or osteomyelitis), was significantly higher than that for carriage isolates (defined as nasal carriage + and orthopaedic implant-associated isolates), (84.4% versus 68.4%, respectively;  $p = 0.023$ , Fisher's exact test).

The inclusion of implant-associated isolates in the carriage group was based on the assumption that deep wound infections are caused by entry of colonising flora at the time of surgery. A second comparison of nasal carriage isolates with the three remaining groups combined gave an equivalent result, (64% and 82%, respectively;  $p = 0.019$ , Fisher's exact test).

### **5.3.6 The number of *fnb* genes does not correlate with overall fibronectin binding.**

The adherence of 163 clinical isolates was compared with the number of *fnb* genes on Southern dot blot. There was no significant difference in adherence between isolates with one or two genes (Table 5.3). However, the number of isolates demonstrating poor adhesion, (less than 20% of that for *S. aureus* 8325-4) was significantly greater in isolates with one, compared to two, *fnb* genes (7/37 and 1/126, respectively,  $p = 0.0003$ , Fisher's exact test).

**Table 5.3 Number of *fnb* genes versus adherence to fibronectin for 163 clinical isolates of *S. aureus*.**

	% adherence compared with 8325-4	
	1 <i>fnb</i> gene	2 <i>fnb</i> genes
Median	95.0	99.8
Range	6.9-124.7	4.2-128.2
Interquartile range (25%-75%)	79.9-108.3	89.7-106.0

There was no significant difference in adherence between isolates with one or two *fnb* genes (p = 0.40, Kruskal-Wallis test).

Re-analysis of the data after exclusion of the 8 strains that exhibited poor adherence to fibronectin gave a p value of 0.35.

## 5.4 Discussion.

This study has undertaken an evaluation of the FnBPs of 163 clinical isolates of *S. aureus*. These were collected from two geographically distant countries to reduce the chance of studying organisms derived from a restricted number of clones. Organisms were selected on the basis of clinical history, but were otherwise chosen by taking consecutive methicillin-susceptible isolates identified in the laboratory in a given clinical group. Orthopaedic implant-associated infection was used as a model of deep wound infection because the use of multiple criteria, (the clinical picture, positive histology in the absence of inflammatory joint disease and positive culture of multiple deep specimens), increases the reliability of a diagnosis of infection (Atkins *et al.*, 1998). This compares favourably with other infections (eg. superficial surgical wound infection, leg ulcer-associated cellulitis), where the diagnosis is often made on clinical grounds in association with a single surface swab that is culture positive for *S. aureus* (Waldvogel, 2000).

A microtitre assay was used to evaluate bacterial adherence to purified human fibronectin *in vitro*. The demonstration of an approximately linear relationship between OD<sub>405</sub> and the number of adherent bacteria (as shown in Chapter 2) facilitated the use of this assay to compare adherence between isolates. Comparison between the four groups as defined by clinical history demonstrated that adherence of those associated with orthopaedic implant-related infection was greater than that for the other three groups. Bone-implanted metallic devices become coated with fibronectin in animal models (Fischer *et al.*, 1996), and it is possible that efficient adherence to this host protein is an important factor in the pathogenic process. The reservoir of *S. aureus* for wound-related infection is predominantly that of the patient's endogenous flora (Wenzel & Perl, 1995), and adherence might, therefore, be expected to mirror that of nasal carriage isolates. This is clearly not the case, and raises the question as to whether a process of *in vivo* selection has taken



place in which efficient adherence to fibronectin is associated with an enhanced pathogenic potential. Isolates that adhered poorly were, however, present in all three groups associated with clinical disease including community-acquired infection. Hence it appears that functional FnBPs are not essential for the survival of organisms during invasive disease. Studies of deficient mutants in animal models support the view that disease is sustainable in the absence of FnBPs (Flock *et al.*, 1996; Kuypers & Proctor, 1989).

Taken overall, adherence of 155/163 isolates ranged from 56% to 124.7% of that of *S. aureus* 8325-4. This degree of variation has previously been reported for adherence of clinical isolates to collagen (Thomas *et al.*, 1999) and fibronectin (Switalski *et al.*, 1983), and is not unexpected given the likely genetic heterogeneity within the bacterial population. The reason for the poor adherence of 8/163 isolates was evaluated. It seems unlikely that this resulted from proteolysis of FnBPs since there was no difference in adherence after culture in the presence of an universal protease inhibitor. Two of 8 strains had bands consistent with fibronectin-binding proteins on Western ligand affinity blot which were of the same intensity as that for good binders. It is possible that poor binding in these two isolates was due to conformational effects when expressed on the bacterial cell surface. For example, there could be masking of FnBP ligand binding regions through incorrect protein folding or interference from other surface components, or the D region of FnBP may be buried in the peptidoglycan as a result of a truncated wall-spanning region. The Western blot did not demonstrate functional expression of FnBPs for 6 poor binders. The mean adherence for these was 10% of 8325-4, and it is possible that this level of binding was mediated via non-specific mechanisms in the absence of FnBPs. The molecular explanation for the negative phenotype could be no/poor transcription of *fnb* genes, no/poor translation of FnBP proteins, expression of mutant FnBP that cannot bind fibronectin detectably by ligand affinity blotting, or defective sorting/anchoring of the FnBP.

Three quarters of the 163 isolates had two genes by dot blot and the remainder had one detectable gene. No isolate lacked both genes. This is consistent with a more recent study which divided 62 isolates into 5 polymorphic groups based on number of *fnb* genes plus the number of D repeats in each D region (Rice I <sup>et al. 2001a</sup> ~~and I 2001-69-3791~~). The majority (>70%) fell into a group with two genes, each of which had three D repeats. Studies with site-specific *fnbA* and *fnbB* insertion mutants of *S. aureus* 8325-4 have shown that either FnBPA or FnBPB can mediate adherence to fibronectin-coated coverslips, and that there is no significant difference in adherence between the wild-type and single *fnb* mutants (Greene *et al.*, 1995). This concurs with the results of this dissertation in which adherence to fibronectin did not differ between isolates with one or two *fnb* genes.

*S. aureus* isolates associated with invasive disease were significantly more likely to have two *fnb* genes. The explanation for this observation is unclear, but may be due to the presence of other virulence determinants that are associated with a genotype containing two FnBP genes. Isolates with one gene were over-represented in the low adherence group and although only one gene is required for efficient adhesion to fibronectin, two genes may confer protection against loss of functional FnBP expression following a significant mutation in a single *fnb* gene.

Stationary phase bacteria were used for the adherence assays. This represents a potential criticism of the methodology since *in vitro* studies have demonstrated that FnBPs are synthesised during the early exponential phase of growth (Saravia-Otten *et al.*, 1997), and may become modified during stationary phase by staphylococcal proteases (McGavin *et al.*, 1997). It is difficult to predict which phase is relevant to human disease, since the predominant phase of bacterial growth and the pattern of FnBP expression and protease secretion *in vivo* is not known. However, it has recently been shown that RNAIII, the mediator of the *agr* response, is expressed early by *S. aureus* following

aggregation with plasma proteins (J. Lindsay, personal communication), suggesting that study of function in stationary phase is important. In addition, the effect of V8 protease secreted during stationary phase on FnBPs varies between isolates. McGavin *et al.* clearly demonstrated that V8 protease was responsible for FnBP degradation in a single selected clinical isolate (McGavin *et al.*, 1997). He also reported that variation in fibronectin binding among 44 MRSA isolates was inversely related to protease activity (Rice *et al.*, 2001a). However, V8 protease was not responsible for degradation of this adhesin in V8 protease-defective isogenic mutants constructed in two different genetic backgrounds (see Chapter 6).

## **5.5 Chapter summary.**

This study has demonstrated diversity in adherence to fibronectin and in the number of genes encoding FnBPs in a large population of clinical isolates. There was no difference in adherence between isolates with one or two *fnb*, but isolates associated with invasive disease were more likely to have two genes. In addition, adherence of the bacterial group associated with orthopaedic implant infection was significantly greater than that for other isolate groups. These findings suggest that the interplay between pathogenesis and a single virulence determinant is unlikely to be a uniform process across a spectrum of infections and hosts. This confirms the need to extend the study of staphylococcal pathogenesis from the laboratory to real, albeit non-uniform populations of clinically relevant isolates.

## **Chapter 6. Adherence of *S. aureus* 8325-4 and *S. aureus* V8 to fibronectin is not modified by serine (V8) protease.**

### **6.1 Chapter content.**

Proteases secreted by *S. aureus* may influence cell wall associated adhesins of the bacterium through a process of degradation. This chapter examines the effect of serine (V8) protease on the fibronectin binding phenotype of *S. aureus*. This was achieved by:

- 1 Construction of *S. aureus* mutants defective in the gene encoding serine (V8) protease. These were verified at genotypic and phenotypic levels using Southern hybridization, Western immunoblotting and total protease assay.
- 2 Comparison between mutant and wild-type of:
  - i. FnBP expression using Western blotting.
  - ii. Adherence to fibronectin using a microtitre plate assay.
  - iii. The interaction with human endothelial cells *in vitro*.

## 6.2 Materials and methods.

### 6.2.1 Construction of an *sspA::Tc<sup>R</sup>* mutant.

Two internal fragments of the gene encoding serine (V8) protease, hereafter called *sspA* (staphylococcal serine protease A), were amplified by PCR from *S. aureus* 8325-4 chromosomal DNA. A 754 b.p. fragment (corresponding to bases 4-758) was amplified using the following primers: CCCATCGATTTGTTCTTCGAAACTTAAGC and CCCAAGCTTAGGGAATGCTTTTAAAGC, which introduced a *Cla*I restriction site at the 5' end of the fragment and a *Hind*III restriction site at the 3' end of the fragment. A 796 b.p. fragment (corresponding to bases 759-1555) was also amplified using the following primer set: CCCAAGCTTTCTGCAATTAACCAAGAC and CGCGGATCCTCGGCTTTGGCTTTATTG, which introduced a *Hind*III restriction site at the 5' end of the fragment and a *Bam*III restriction site at the 3' end of the fragment. The magnesium concentration used was 1.5 mM, and amplification consisted of 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Aliquots of the reaction mixtures were analyzed by 1% agarose gel electrophoresis. The fragments were purified, ligated at the *Hind*III site and cloned into pBluescript at the *Bam*HI and *Cla*I sites. The resulting plasmid was linearized by digestion with *Hind*III and ligated with a 2.1kb *Hind*III fragment from pCW59 that confers tetracycline resistance. A shuttle plasmid was constructed by combining the resulting plasmid with the *S. aureus* temperature sensitive plasmid pTS2 (*ts*, chloramphenicol resistant). The shuttle plasmid was electroporated into *S. aureus* 8325-4 following transformation into the restriction negative host, RN4220. Temperature shift experiments resulted in the isolation of an allelic replacement mutant in *sspA* of 8325-4 (strain DU5956). The 8325-4 *sspA::Tc<sup>R</sup>* mutant was transduced by phage 85 (Asheshov, 1966) into *S. aureus* strain V8 forming DU5957.

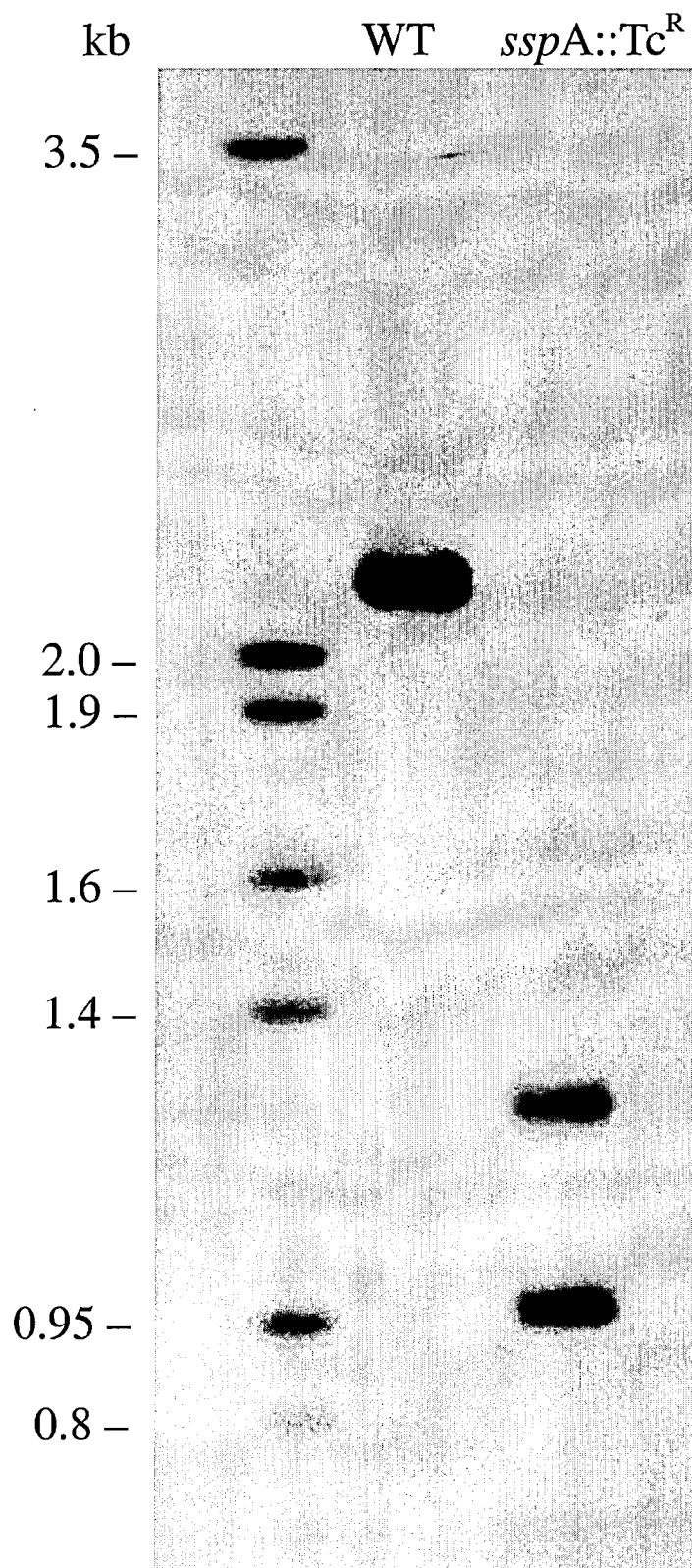
## 6.3 Results.

### 6.3.1 Verification of the mutant genotype.

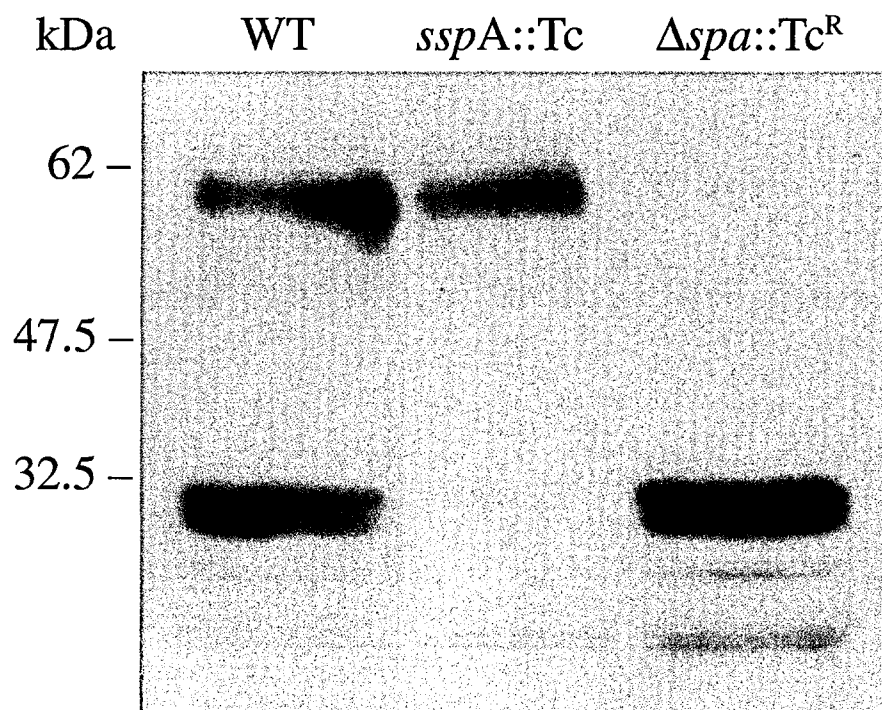
The chromosomal *sspA::Tc<sup>R</sup>* mutation was verified by Southern hybridization. Chromosomal DNA from the wild-type and *sspA::Tc<sup>R</sup>* mutant was isolated, digested with *Hind*III and probed using a DIG-labelled probe generated by PCR and specific to the full length gene using primers and conditions described above, with the exception of the elongation time which was 1 min 30s. The product was purified from 1% agarose gel using a DNA extraction kit and DIG-dUTP labelled by PCR. A single band of approximately 2.1kb hybridized from the wild-type parent (Fig. 6.1). Two fragments of approximately 1.2kb and 950bp hybridized from the 8325-4 *sspA::Tc<sup>R</sup>* mutant, showing that the full-length gene had been separated by the introduced *Hind*III site (Fig. 6.1). The 8325-4 *sspA::Tc<sup>R</sup>* mutant was transduced into *S. aureus* strain V8, and the mutation verified by Southern hybridization.

### 6.3.2 Verification of the mutant phenotype.

The presence of SspA in the supernatant was analysed by Western immunoblotting. Isolates were inoculated from frozen stocks into 10ml of TSB and incubated for 15 to 18 hours under constant rotation at 37°C in air. The culture was centrifuged for 10 minutes at 3,000 rpm and the supernatant concentrated 20 times using a Centricon-10 (Amicon). This was analysed by Western immunoblotting using rabbit polyclonal anti-V8 protease antibody (1:2000, a gift from Dr S. Arvidson), followed by peroxidase-conjugated goat anti-rabbit immunoglobulin (1:2000, Dako). Figure 6.2 demonstrates the loss of an immunoreactive band with an apparent molecular mass of approximately 30kDa from the supernatant of the *sspA::Tc<sup>R</sup>* 8325-4 mutant compared with the parent strain. This is consistent with the loss of SspA production.



**Figure 6.1 inactivation of *S. aureus* *sspA*.** Southern hybridization blot of *Hind*III-digested chromosomal DNA from parent strain 8325-4 (WT) and its *sspA*::Tc<sup>R</sup> mutant, probed with a PCR-amplified full length *sspA* gene. Marker sizes in kilobases are shown on the left.



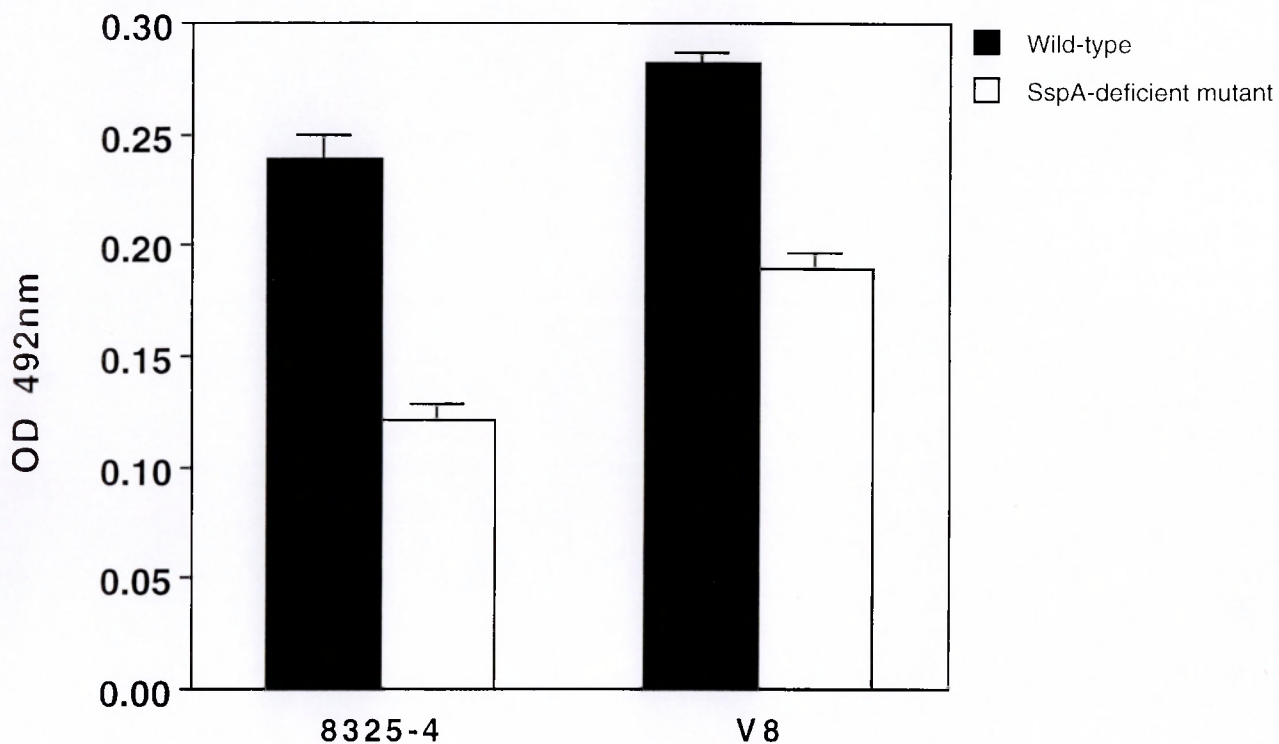
**Figure 6.2 Visualization of SspA by Western immunoblotting of *S. aureus* culture supernatant.** Lane 1, 8325-4; lane 2, 8325-4 DU5723 defective in protein A; lane 3, 8325-4 *sspA::Tc<sup>R</sup>*. Molecular mass markers (on the left) are in kilodaltons. The loss of an immunoreactive band with an apparent molecular mass of approximately 30kDa is shown for 8325-4 *sspA::Tc<sup>R</sup>*, consistent with the loss of SspA production. The ~55kDa band was absent from the culture supernatant of a protein-A defective 8325-4 mutant (DU5723).



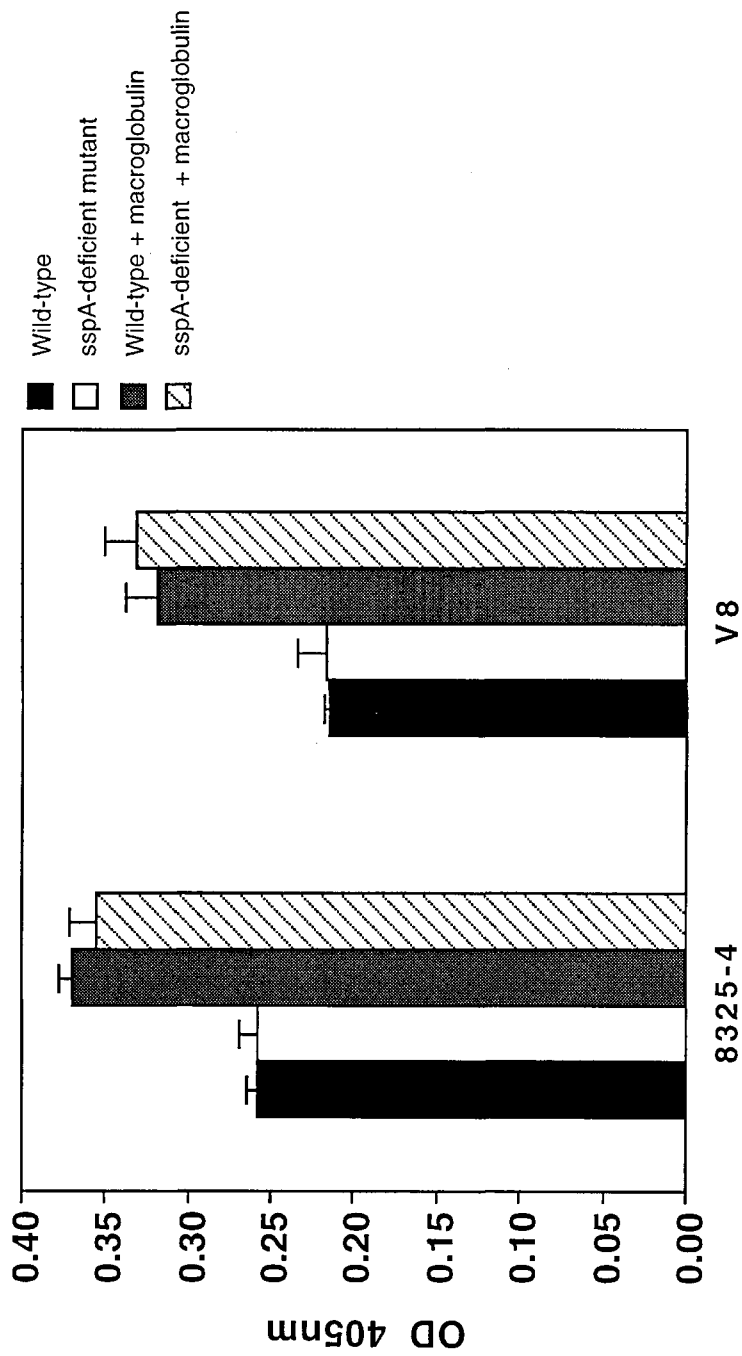
Quantitative determination of total protease activity in 20-fold concentrated culture supernatants was performed using a protease assay kit. Assays were performed in triplicate for 8325-4 and V8 together with their respective SspA-deficient mutants. Protease activity of the 8325-4 *sspA::Tc<sup>R</sup>* and V8 *sspA::Tc<sup>R</sup>* mutants was reduced by 51% and 67%, respectively, compared with the parent strain (Fig. 6.3). This indicates that these isolates produce other proteases in addition to SspA.

### **6.3.3 Effect of *sspA* mutation on adherence of *S. aureus* to fibronectin *in vitro*.**

Adherence of *S. aureus* to purified human fibronectin 10µg/ml was assessed using a standardised microtitre plate assay. Each isolate was tested in quadruplicate in an individual assay and each experiment was performed three times. Adherence of *S. aureus* 8325-4 and V8 to fibronectin was compared with that of the SspA-deficient mutants. The production of SspA during overnight culture did not affect bacterial adherence to fibronectin ( $p = 0.97$  and  $0.94$  for 8325-4 and V8 respectively, paired t-test, Fig. 6.4). Following culture in the presence of the universal protease inhibitor  $\alpha_2$ -macroglobulin 0.125U/ml (Boehringer Mannheim), 8325-4 and V8, together with their respective SspA-deficient mutants, all demonstrated a significant increase in adherence to fibronectin ( $p < 0.05$  in all cases). This suggests that adherence of 8325-4 and V8 to fibronectin is modified by a protease other than SspA.



**Figure 6.3 Total protease activity of *S. aureus* strains 8325-4 and V8 compared with their respective SspA-deficient mutants.** The assay was based on cleavage of fluorescein thiocarbamoyl-casein derivative by proteases as detected by a change in absorbance. Protease activity was evaluated using 20-fold concentrated supernatants from overnight broth culture. Results are shown for the mean  $\pm$  SEM.



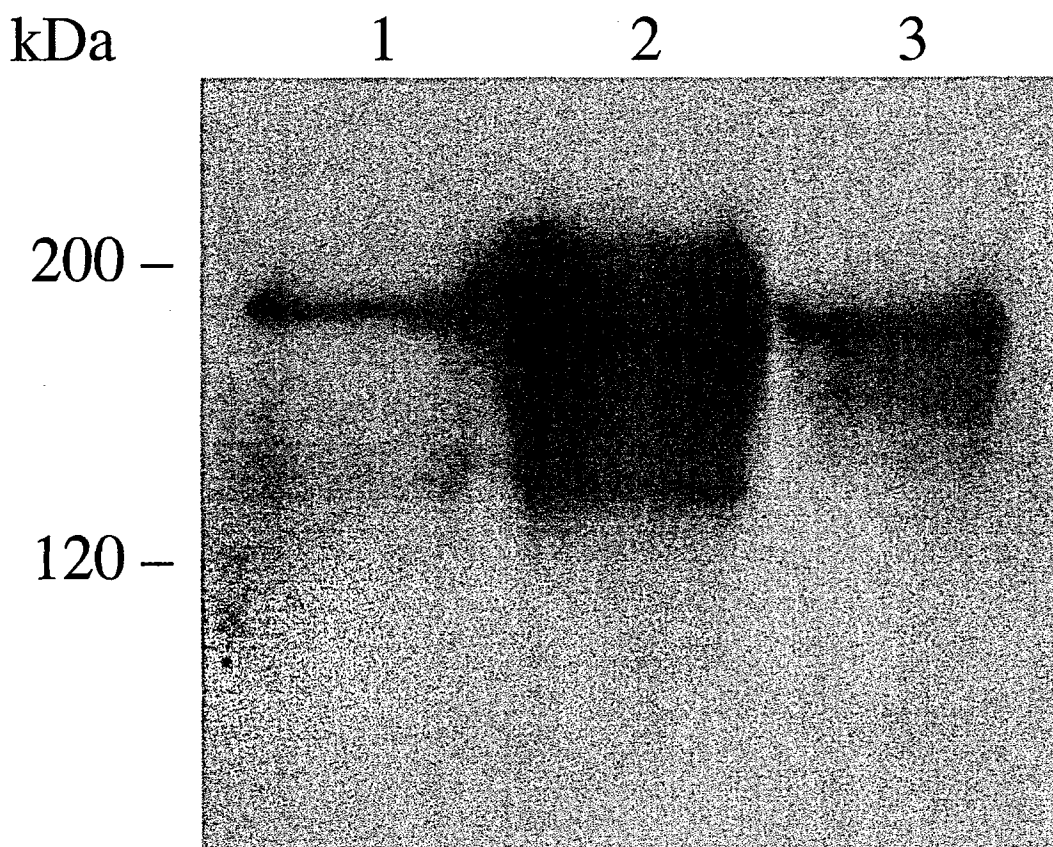
**Figure 6.4 Adherence of *S. aureus* to fibronectin *in vitro*.** Adherence of *S. aureus* strains 8325-4 and V8 and their respective SspA-deficient mutants was evaluated using a standardised microtitre assay following overnight culture in the presence or absence of the universal protease inhibitor  $\alpha_2$ -macroglobulin at 0.125U/ml. Results are shown for the mean  $\pm$  SEM.

#### 6.3.4 Effect of *sspA* mutation on fibronectin-binding proteins.

Cell wall-associated proteins were isolated from standardised inocula of *S. aureus* 8325-4 and the 8325-4 *sspA*::Tc<sup>R</sup> mutant. Equal amounts of cell wall-associated protein extract were separated by SDS-polyacrylamide gel electrophoresis and evaluated by Western ligand affinity blotting. A protein with an apparent molecular mass of approximately 180kDa (consistent with fibronectin-binding protein) was present in all extracts. The intensity of the band was comparable between 8325-4 wild-type and the 8325-4 *sspA* mutant (Fig. 6.5). A comparison was made between 8325-4 cultured in the presence or absence of  $\alpha_2$ -macroglobulin 0.125U/ml. Growth in the presence of  $\alpha_2$ -macroglobulin resulted in a marked increased in band intensity (Fig. 6.5). This indicates that while degradation of the FnBP of *S. aureus* 8325-4 occurs during culture, the process is mediated by a protease other than SspA.

#### 6.3.5 Effect of *sspA* mutation on *S. aureus* adherence to, and uptake by endothelial cells *in vitro*.

Adherence of *S. aureus* to, and uptake by endothelial cells *in vitro* is mediated by the interaction between bacterial fibronectin-binding protein and endothelial cell-surface fibronectin (Peacock *et al.*, 1999). The effect of SspA on this interaction was assessed using the standardised endothelial cell adherence assay as previously described. The number of bacteria either associated with (adherent + intracellular), or internalised by 1mm<sup>2</sup> of confluent endothelial cells was not significantly different between 8325-4 and the 8325-4 *sspA*::Tc<sup>R</sup> mutant ( $p > 0.05$  for both assays, paired t-test).



**Figure 6.5 Visualization of fibronectin-binding proteins by Western ligand affinity blotting of cell wall-associated protein extracts.** Equal amounts of cell wall-associated protein extract were loaded into each lane. *S. aureus* 8325-4 following culture in the absence (lane 1), or presence of  $\alpha_2$ -macroglobulin at 0.125U/ml (lane 2); lane 3, 8325-4 *sspA::Tc<sup>R</sup>* mutant following culture in the absence of  $\alpha_2$ -macroglobulin. Molecular mass markers (on the left) are in kilodaltons.

## 6.4 Discussion.

Serine (V8) protease was first purified from the culture supernatant of *S. aureus* strain V8 in 1972, and found to specifically cleave peptide bonds on the carboxyl terminal side of aspartate or glutamate (Drapeau *et al.*, 1972). Two mutants with increased protease production isolated after chemical mutagenesis were described the following year, the phenotypes of which were markedly different from that of wild type in terms of carbohydrate utilisation, and altered patterns of extracellular alkaline phosphatase and deoxyribonuclease (Ryden *et al.*, 1973). In retrospect, these pleiotropic changes were probably related to a mutation in one of the staphylococcal global regulators, since synthesis of serine protease is now known to involve positive regulation by *agr* and negative regulation by *sarA* (Chan and Foster 1998). This is reflected by the findings that serine protease is expressed in the post-exponential phase (consistent with up-regulation by *agr*), and that production is increased in a *sarA* defective mutant (Chan & Foster, 1998). Availability of *S. aureus* sequence data (an event occurring after completion of the work for this chapter), has since added further to our understanding of serine protease expression. Sequence analysis of the *S. aureus* strain COL genome reveals that the *ssp* gene encoding serine protein<sup>ase</sup> (*sspA*) is followed closely by an open reading frame (ORF) encoding a cysteine protease designated *sspB* (Rice *et al.*, 2001b). The *sspA* and *sspB* proteases are transcribed as an operon, which also includes a third ORF *sspC* of unknown function. A nonpolar allelic replacement mutation of *sspA* resulted in loss of autolysin activity and proteolytic maturation of the SspB cysteine protease, indicating that SspA controls SspB (Rice *et al.*, 2001b). Furthermore, SspA is itself expressed as an inactive precursor that is activated by a metalloprotease (Drapeau *et al.*, 1972).

Signature tagged mutagenesis has shown that serine protease contributes to *in vivo* growth and survival of *S. aureus* (Coulter *et al.*, 1998), although inactivation of *sspA* did not result in attenuation in a tissue abscess

model of infection (Rice *et al.*, 2001b). So what role, if any, does SspA play in modelling cell surface-expressed adhesins? Since the work for this thesis was completed, two papers have been published which add to the findings described here. The first, by Rice *et al.* in January 2001, recognised the potential importance of nonpolar mutations in relation to *ssp* genes and constructed an *sspA* mutant without affecting *sspB* or *sspC* (Rice *et al.*, 2001b). Binding to <sup>125</sup>I labelled fibronectin did not differ between the mutant and wild type in either mid-exponential or stationary phase. These findings are in agreement with those of this thesis, although it should be noted that the strain constructed during this work was probably defective in all three genes contained on the *ssp* operon. The second publication in August 2001 reported the effects of mutating the *ssp* operon by allelic replacement in 8325-4, finding that there was a three-fold increase in cell-bound FnBPs as detected by Western immunoblotting (Karlsson *et al.*, 2001). Comparison of fibronectin adherence between mutant and wild type strains was not ~~was not~~ reported by these investigators, making it difficult to directly compare with this thesis. It is possible that cell wall fractions from *ssp* mutants contain more FnBP than wild-type, but that this can only be detected by the more sensitive immunoblotting technique (as compared with ligand affinity blotting used during this chapter, a choice based on the lack of anti-FnBP antibodies at that time). However, it remains to be seen whether this difference translates to an effect on function.

## 6.5 Chapter summary.

This study has demonstrated that FnBP expressed by *S. aureus* 8325-4 and the adherence of 8325-4 and V8 to fibronectin is not modified by SspA. *S. aureus* FnBP expression and bacterial adherence to fibronectin was increased for wild-type 8325-4 and V8 and their SspA-deficient mutants following growth in the presence of the universal protease inhibitor  $\alpha_2$ -macroglobulin. Thus,

degradation of FnBPs during growth of *S. aureus* V8 and 8325-4 appear to be mediated by a protease other than SspA.



## **Chapter 7. *Staphylococcus schleiferi* subsp. *schieiferi* expresses a fibronectin-binding protein.**

### **7.1 Chapter content.**

*S. schleiferi* is a nosocomial pathogen that causes infection primarily in association with prosthetic material. Bacterial adhesins recognizing fibronectin and fibrinogen play a central role in the pathogenesis of colonization of medical devices by *S. aureus* (Vaudoaux *et al.*, 1989, 1993, 1995). *S. schleiferi* has been reported to bind fibrinogen as assessed by commercial agglutination kits (Hébert, 1990; Personne *et al.*, 1997; Vandenesch *et al.*, 1994), but adherence to fibronectin and the identification of cell wall-associated adhesins have not been described for this organism. This study set out to investigate the possibility that *S. schleiferi* expresses a fibronectin-binding protein. This was achieved by:

- 1      Determining adherence of laboratory and clinical *S. schleiferi* isolates to fibronectin, and examining the effect on adherence of recombinant FnBP protein and removal of surface proteins with proteinase K.
- 2      Examining *S. schleiferi* fibronectin-binding protein by Western ligand affinity blot.
- 3      Amplifying (by PCR) and sequencing a fragment of the gene encoding the fibronectin-binding protein of *S. schleiferi*.

## 7.2 Bacterial strains.

*S. schleiferi* NCTC 12218 and *S. epidermidis* NCTC 11047 were obtained from the National Collection of Type Cultures, U.K. Twenty five clinical isolates of *S. schleiferi* were obtained from the Centre Nationale de Reference des Toxemies à Staphylocoques, Lyon, France. These were isolated from: blood culture (n=11); pacemaker lead, device or wound (8); intravenous cannula (1); brain abscess (1); cerebrospinal fluid (1); and wound infections (3).

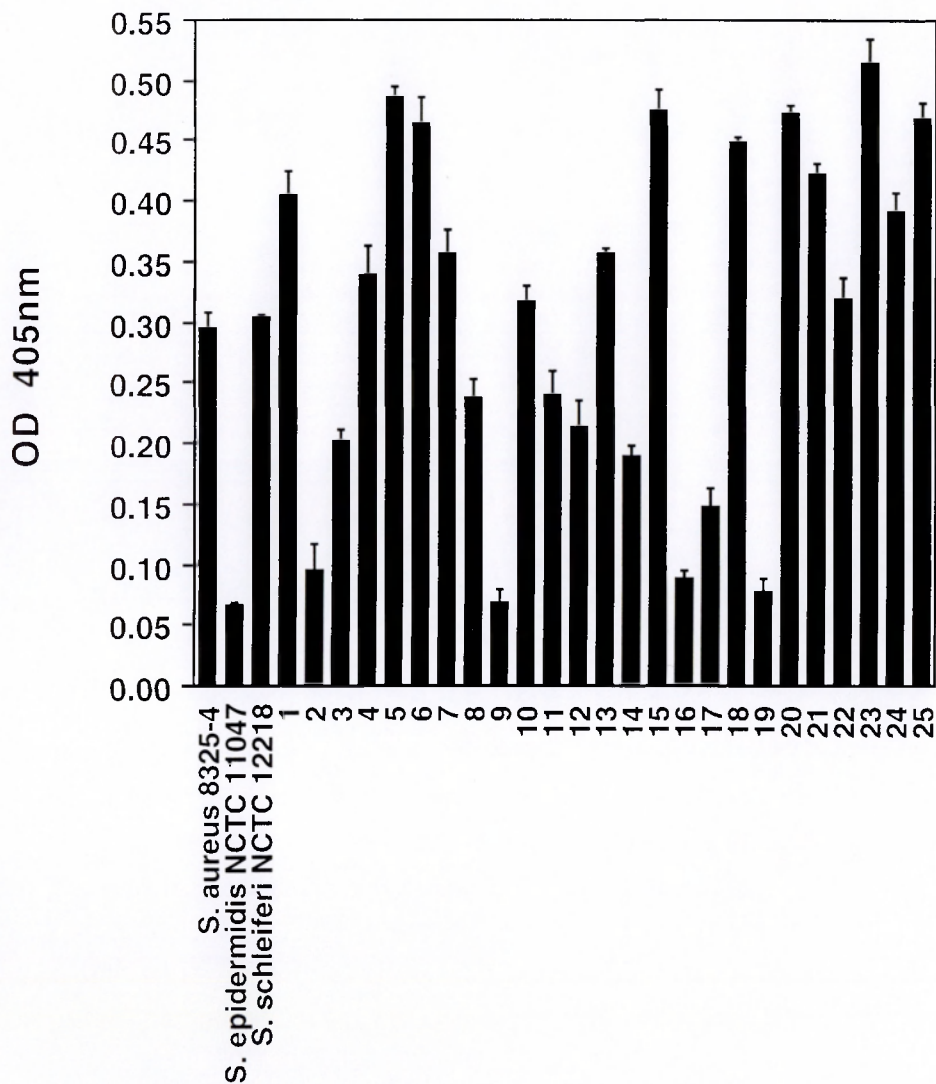
### 7.3 Results.

Experimental results for microtitre plate adherence assays are shown throughout as the mean of triplicate experiments (each isolate being tested in quadruplicate in an individual assay)  $\pm$  standard error of the mean.

#### 7.3.1 *S. schleiferi* adheres to purified fibronectin *in vitro*.

Adherence of bacterial isolates to purified human fibronectin 10 $\mu$ g/ml was assessed using a microtitre plate assay. Adherence of *S. schleiferi* NCTC 12218 to purified fibronectin was compared with *S. aureus* 8325-4 (positive control), and with *S. epidermidis* NCTC 11047 which adheres poorly. The ability of *S. schleiferi* to adhere to purified fibronectin *in vitro* was demonstrated (Fig. 7.1). The level of binding was comparable to the positive control, with no significant difference in OD<sub>405</sub> for *S. schleiferi* compared with that for *S. aureus* 8325-4 ( $p = 0.45$ , unpaired t-test). The adherence of *S. schleiferi* and *S. aureus* was significantly greater than that for *S. epidermidis* ( $p < 0.0001$  for both strains, unpaired t-test). Equivalence of bacterial inocula ( $1 \times 10^8$  cfu per well) for the three strains was confirmed.

To evaluate whether recent isolates of *S. schleiferi* associated with disease were also able to adhere to fibronectin, adherence assays were performed for 25 clinical isolates. All isolates adhered to fibronectin, but there was marked variation in OD<sub>405</sub> values within the group, with a 7.4 fold difference in absorbance between the lowest and highest binders (Fig. 7.1). This variation was highly reproducible, as reflected by the values for the standard error of the mean (Fig. 7.1). Colony count experiments excluded variation in bacterial inocula as the cause.



**Figure 7.1 Adherence of *S. schleiferi* to purified human fibronectin *in vitro*.** Adherence was compared between *S. aureus* 8325-4, *S. epidermidis* NCTC 11047, *S. schleiferi* NCTC 12218 and 25 clinical isolates of *S. schleiferi* from the following microbiological specimens: blood culture (numbered 1-11); pacemaker lead, box or wound (nos. 12-19); intravenous cannula (no. 20); brain abscess (no. 21); cerebrospinal fluid (no. 22); and wound infection (nos. 23-25).

### **7.3.2 Adherence to fibronectin is inhibited by the recombinant form of the ligand binding region of *Streptococcus dysgalactiae* FnBPB.**

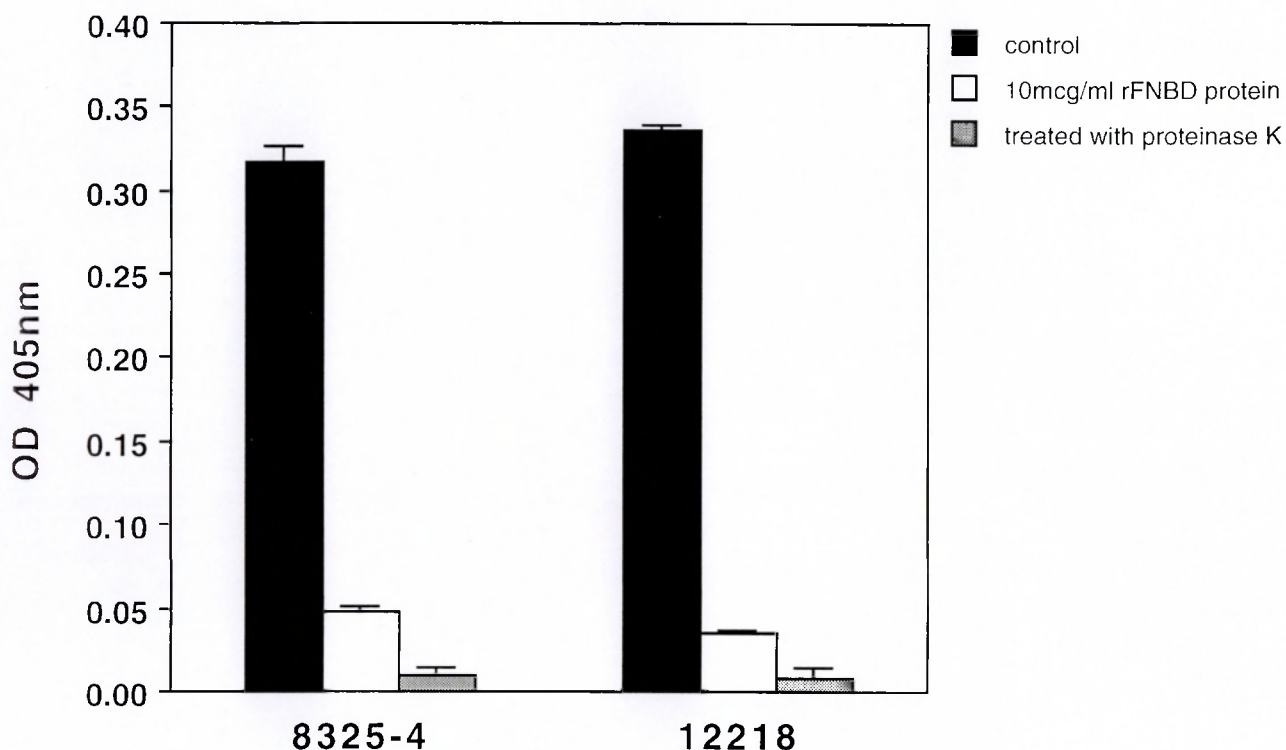
The microtitre plate assay was repeated for *S. aureus* 8325-4 and *S. schleiferi* NCTC 12218 in the presence of the recombinant form of the ligand binding region of *Streptococcus dysgalactiae* FnBPB (Joh *et al.*, 1994) (rFNBD-B, a gift from Dr. Magnus Höök, Texas, referred to below as rFNBD protein). This was added to the wells immediately prior to bacterial inoculation at a final concentration of 10µg/ml. Control wells were incubated with bacteria in the absence of rFNBD protein. The total volume was maintained at 100µl for all wells.

*S. aureus* adherence was significantly reduced in the presence of rFNBD protein (15% of control,  $p = 0.0002$ , paired t-test) (Fig. 7.2), consistent with published data (Joh *et al.*, 1994). *S. schleiferi* adherence was also significantly reduced in the presence of rFNBD protein (10% of control,  $p < 0.0001$ , paired t-test). This provides further evidence for the expression of a cell surface-associated fibronectin-binding protein by *S. schleiferi* that has homology to rFNBD protein, or whose function is sterically hindered by its presence.

### **7.3.3 Adherence of *S. schleiferi* NCTC 12218 to purified fibronectin is significantly reduced by treatment with proteinase K.**

Surface proteins were removed from the bacterial pellet of 10ml overnight cultures of *S. schleiferi* NCTC 12218 and *S. aureus* 8325-4 using proteinase K. Adherence to purified human fibronectin was compared between organisms pre-treated with proteinase K and untreated controls (Fig. 7.2). Proteinase K treatment resulted in a highly significant reduction in adherence for both *S. schleiferi* NCTC 12218 ( $p < 0.0001$ ), and *S. aureus* 8325-4 ( $p = 0.0002$ , paired t-test). The results for *S. aureus* are consistent with the proteolytic degradation of fibronectin-binding protein. The observation that binding of *S.*

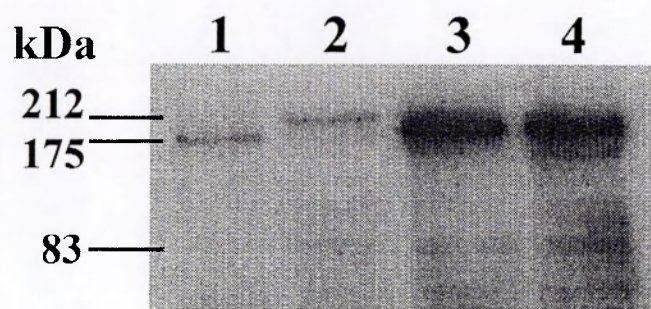
*schleiferi* to fibronectin was almost abolished following exposure to proteinase K suggests that adherence of this organism to fibronectin is also mediated by a cell surface-expressed protein.



**Figure 7.2 Effect of proteinase K and the recombinant form of the ligand binding region of *Streptococcus dysgalactiae* FnBPB on adherence to purified human fibronectin *in vitro*.** Adherence of *S. schleiferi* NCTC 12218 and *S. aureus* 8325-4 was evaluated: (i) following treatment with proteinase K; and (ii) in the presence of 10µg/ml of the recombinant form of the ligand binding region of FnBPB (rFNBD protein) encoded by *fnbB* of *Streptococcus dysgalactiae*.

#### 7.3.4 *S. schleiferi* express a fibronectin-binding protein on Western ligand affinity blot.

Cell wall-associated protein extracts were prepared from *S. aureus* 8325-4 and *S. schleiferi*, separated by SDS-polyacrylamide gel electrophoresis (PAGE) and evaluated by Western ligand affinity blotting. *S. aureus* 8325-4 was used as a positive control and was shown to express a protein with an apparent molecular mass of approximately 180kDa (Fig. 7.3), consistent with previous reports ((Bisognano *et al.*, 1997; Greene *et al.*, 1995; Vaudaux *et al.*, 1998). The extracts from *S. schleiferi* NCTC 12218 and two randomly selected clinical *S. schleiferi* isolates (numbered 3 and 18 in Fig. 7.1) also contained a reactive protein band (Fig. 7.3). That for NCTC 12218 had a molecular mass of approximately 200kDa. The bands for the two clinical *S. schleiferi* isolates were of greater density on visual inspection compared both with *S. schleiferi* NCTC 12218 and *S. aureus* 8325-4. Western ligand affinity blot was repeated using a 1 in 4 dilution of the initial cell wall extract from the clinical isolates to facilitate assessment of the molecular mass of these positive bands. This demonstrated the reactive bands to be 180kDa for both clinical isolates (data not shown). The greater band density for the two clinical isolates using the original extract occurred despite standardization of the bacterial inoculum used during cell wall-associated protein extraction, and was reproducible on repeat testing with an independently prepared protein extract (data not shown). However, greater band density did not predict an enhanced adherence to fibronectin *in vitro*, the adherence of strain 18 being greater than, and that for strain 3 being less than NCTC 12218 (Fig. 7.1).



**Figure 7.3 Visualization of fibronectin-binding proteins by Western ligand affinity blot of cell wall-associated protein extracts.** Equal amounts of cell wall-associated protein extract were loaded into each lane. Lane 1, *S. aureus* 8325-4; lane 2, *S. schleiferi* NCTC 12218; lanes 3 and 4, clinical *S. schleiferi* isolates, (numbered 18 and 3 in figure 7.1, respectively). Molecular mass markers (on the left) are in kilodaltons.



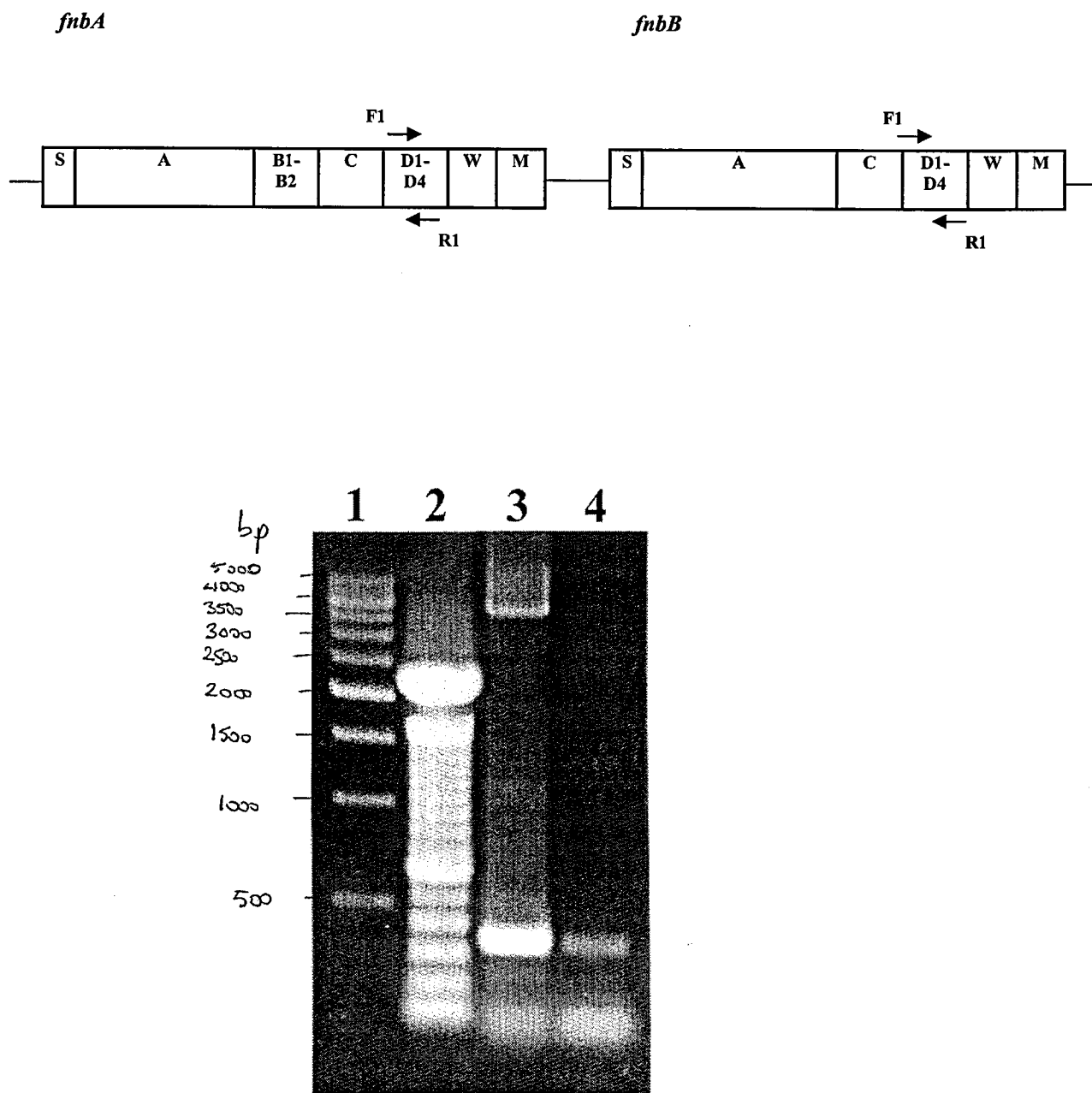
### 7.3.5 PCR amplification and sequencing of a fragment of *S. schleiferi* *fnb*.

PCR analysis was performed to determine the presence of homology between primers to the D1-D3 binding region of *S. aureus* 8325-4 *fnbA* and genomic DNA from *S. schleiferi* NCTC 12218. The rationale for this choice of primer site was that the D-repeat region is highly conserved between the *fnbA* and *fnbB* of *S. aureus* 8325-4, probably as a result of functional requirements of the binding domains, and represents the area of greatest homology between FnBPs of distantly related species (Joh *et al.*, 1994). Oligonucleotide primers were synthesized by Genosys, as follows: 5'-GGCCAAAATAGCGGTAACC-3' (forward) and 5'-GCTTACTTTTGGAAAGTGTATC-3' (reverse) corresponding to bases 2349-2367 and 2674-2694 of *fnbA*, respectively (as assigned by the GenBank sequence). The magnesium concentration used was 1.5 mM, and amplification consisted of 30 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 3 min. Agarose gel electrophoresis of an aliquot of the PCR reaction mixtures is shown in Figure 7.4.

A predictable band of approximately 350bp was seen for *S. aureus* 8325-4, together with a second band of approximately 3.7kb. The primer recognition sites used for *fnbA* had only 1 and 2 base pair differences for forward and reverse primers, respectively between *fnbA* and *fnbB*. Figure 7.4 shows a map of the possible positions at which the *fnbA* primers could anneal in the *fnb* locus. It is likely that the 3.7kb band represents amplification of the region from D1 of *fnbA* to D3 of *fnbB*. The results for *S. schleiferi* shown in lane 4 of Figure 7.4 demonstrate a single band of approximately 350bp.

The 350bp *S. schleiferi* fragment was purified and sequenced with Big-Dye terminator chemistry (ABI Prism) and visualized on an ABI 377 sequencer. This demonstrated 100% homology between the fragment from *S. schleiferi* and region D of *S. aureus* 8325-4 *fnbA* (data not shown). This surprising result was verified by repeat sequencing of a PCR product amplified from newly extracted

DNA from *S. schleiferi* NCTC 12218, all reactions for which were carried out under conditions that ensured the absence of *S. aureus* genomic DNA.



**Figure 7.4 PCR analysis of fibronectin-binding protein gene fragments.** Top diagram shows map of the possible positions at which the *fnbA* primers could anneal in the *S. aureus* 8325-4 *fnb* locus (f denotes forward, and r denotes reverse PCR primer, respectively). Gel image: genomic DNA from *S. aureus* 8325-4 (lane 3) and *S. schleiferi* NCTC 12218 (lane 4) amplified with primers complementary to the 345bp D1-D3 binding region of *S. aureus* 8325-4 *fnbA*. Lane 1, 500-bp molecular size marker; lane 2, 100-bp molecular size marker.

## 7.4 Discussion.

Following the description of *S. schleiferi* subsp. *schleiferi* in 1988 (Freney *et al.*, 1988), this coagulase-negative staphylococcus has been implicated as the causative pathogen in a range of hospital-acquired infections. These include bacteraemia (Jean-Pierre *et al.*, 1989; Latorre *et al.*, 1993), brain abscess (Grattard *et al.*, 1993), pacemaker and other intravenous device-related infections including prosthetic valve endocarditis (Celard *et al.*, 1997; Da Costa *et al.*, 1998; Grattard *et al.*, 1993; Leung *et al.*, 1999), and infections of the urinary tract (Ozturkeri *et al.*, 1994), orthopaedic implants (Jean-Pierre *et al.*, 1989) and surgical wounds (Calvo *et al.*, 2000; Grattard *et al.*, 1993; Hernandez *et al.*, 2001; Kluytmans *et al.*, 1998). The mechanisms by which *S. schleiferi* causes such diseases are unknown, but there is a degree of similarity between the spectrum of infections caused by this microorganism and those associated with *S. aureus*. It is plausible, therefore, that the two species share one or more virulence determinants.

The process by which *S. aureus* colonises prosthetic material has been studied in detail, and has been attributed to the interaction between host fibrinogen and fibronectin that rapidly coats foreign material, and bacterial cell wall-associated adhesins. Isolation of *S. schleiferi* from cultures of prosthetic material suggests the presence of one or more bacterial adhesins with host protein specificity similar to that of *S. aureus*. To date, evaluation of the adherence of *S. schleiferi* to host proteins has been limited to the description of bacterial clumping in the presence of fibrinogen using commercial agglutination kits (Hebert, 1990; Personne *et al.*, 1997; Vandenesch *et al.*, 1994). The role of FnBPs in the colonization of prosthetic material by *S. aureus* led to the hypothesis that *S. schleiferi* expresses a fibronectin-binding protein.

This study has described the adherence of *S. schleiferi* subsp. *schleiferi* NCTC 12218 to purified human fibronectin *in vitro*. The level of adherence was comparable to that of *S. aureus* 8325-4. To confirm that this interaction occurred

in recent clinical strains, adherence was evaluated for *S. schleiferi* isolates associated with a range of significant infections. All 25 isolates tested adhered to fibronectin, but the degree to which this occurred showed variability between the strains. This variability could not be accounted for and the reason remains unclear. Similar observations have been made for the adherence of a collection of clinical strains of *S. aureus* to purified collagen *in vitro* (Thomas *et al.*, 1999). It is possible that there is variation between isolates in the number of cell surface-expressed binding sites, or the efficiency with which FnBPs bind fibronectin, which could in turn be related to the relative genetic heterogeneity that is reported to exist for *S. schleiferi* (Grattard *et al.*, 1993). The small number of isolates in each disease group limits comment on the level of adherence versus clinical diagnosis.

The reduction in adherence of *S. schleiferi* to fibronectin in the presence of the recombinant form of the binding region of *Streptococcus dysgalactiae* FnBPB and following treatment with proteinase K, together with the bands seen on Western ligand affinity blotting using fibronectin as the substrate, provide phenotypic evidence for a bacterial cell surface-expressed fibronectin-binding protein. The visualization of a band from lyostaphin cell wall preparations suggests that this is cell wall-anchored. The reason for the variation in apparent molecular mass of the fibronectin-binding proteins from *S. schleiferi* NCTC 12218 and the two clinical isolates is not apparent. One possibility is that the protein has one or more regions that are characterized by a variable number of tandem repeats. This has been well documented for *S. aureus*, examples being the B-repeat region of the collagen-binding protein (Gillaspy *et al.*, 1998), and the D-repeat region of the fibronectin-binding protein in clinical isolates (Rice *et al.*, 2001a). Protein A variants have also been described that result in variation in apparent molecular mass (Cheung *et al.*, 1987). An alternative explanation is that the protein from the clinical strains underwent degradation before processing, with loss of a small N-terminal fragment.

PCR was utilized during this study to amplify a fragment of genomic DNA from *S. schleiferi* NCTC 12218 using primers to the 345bp region of *fnbA* encoding the D1-D3 binding region of *S. aureus* 8325-4 FnBPA. The product from *S. schleiferi* and the smaller of the two fragments from *S. aureus* were of a comparable size. Sequencing of the *S. schleiferi* fragment provided further evidence for the presence of a fibronectin-binding protein, with a surprising degree of homology (100%) with *S. aureus fnbA*. Suspicious of contamination, PCR and sequencing was repeated under fastidious conditions with newly extracted DNA and a control with reagents but no DNA. The same result was obtained. It is possible that *S. schleiferi* has acquired the *fnb* gene through horizontal transfer from *S. aureus* (or vice versa).

#### **7.5. Chapter summary.**

This study provides phenotypic and genotypic evidence for the expression of a cell wall-associated fibronectin-binding protein by the nosocomial pathogen *S. schleiferi*. Understanding the role of this adhesin in disease pathogenesis requires further study *in vitro* and in animal models. Studies are also needed to clone and sequence the complete *S. schleiferi fnb* gene, details of which may shed light on the origins of this adhesin.

## **Chapter 8. *fnbA* is one of seven genes associated with *S. aureus* virulence.**

### **8.1 Chapter content.**

One of the aims of this study was to compare the presence of the gene encoding *S. aureus* fibronectin-binding protein A in isolates from healthy blood donors and from patients with invasive disease. However, most cases of severe *S. aureus* disease cannot be explained by the action of a single virulence determinant, and it is likely that a number of factors act in combination during the infective process. In view of this, a total of 33 factors were considered in parallel. The basis of the work was to:

- 1      Examine isolates of *S. aureus* associated with community carriage and with both community and hospital-acquired disease in the Oxfordshire area, and study the relationship between disease in man and 33 putative virulence determinants, both individually and in combination.
- 2      Analysis was performed as follows:
  - i        univariate analysis, comparing the presence of bacterial factors in carriage and disease isolates.
  - ii       re-analysis after adjusting for the effect of clonality.
  - iii      analysis of the individual and cumulative effect of virulence-associated determinants.
  - iv      defining whether given combinations of genes were either more or less likely to cause disease.
  - V       exploring the role of horizontal transfer in acquisition of virulence-associated genes.

## **8.2 Materials and Methods.**

### **8.2.1 Bacterial isolates.**

The bacteria examined were 155 isolates recovered from patients with invasive *S. aureus* disease (94 hospital-acquired and 61 community-acquired), and 179 isolates recovered from healthy individuals. These were collected within Oxfordshire, UK between 1997 and 1998 by Dr's Moore, Day and Berendt using a prospective case control design (Peacock *et al.*, 2002). Cases of invasive infection were identified prospectively through the microbiology laboratory serving the main hospitals in the Oxford area. Clinical details were recorded and the bacterial isolate was stored. Community-acquired disease was defined as admission to hospital with an illness consistent with invasive *S. aureus* disease, with isolation of *S. aureus* from a normally sterile site within 24 hours of admission. Hospital-acquired disease was defined as an illness consistent with invasive *S. aureus* disease and isolation of the organism from a normally sterile site 48 hours or more after admission for another condition. Control bacteria were obtained from nasal swabs of blood donors drawn from the same population catchment area as the Oxford Radcliffe Hospitals.

### **8.2.2 Evaluation of bacterial determinants.**

A total of 33 bacterial determinants were evaluated. Twenty nine determinants were examined for the presence of the gene using the polymerase chain reaction (PCR), and four determinants were evaluated by phenotypic tests. The bacterial determinants examined incorporated the major putative virulence factors reported in the published literature at the start of the study (Table 8.1).



Table 8.1 Bacterial determinants examined.

Bacterial determinant	Putative function	Ref.	Method of detection
<b>Adhesins</b>			
FnBPA	Adhesin for fibronectin	(Greene <i>et al.</i> , 1995)	PCR for <i>fnbA</i>
ClfA and ClfB	Adhesins for fibrinogen	(McDevitt <i>et al.</i> , 1994; Ni Eidhin <i>et al.</i> , 1998)	PCR for <i>clfA</i> & <i>clfB</i>
Cna	Adhesin for collagen	(Patti <i>et al.</i> , 1992)	PCR for <i>cna</i>
Protein A	Binds Fc domain of Ig and vWF	(Forsgren <i>et al.</i> , 1983; Hartleib <i>et al.</i> , 2000)	PCR for <i>spa</i>
SdrC, SdrD, SdrE	Unknown; putative adhesins	(Josefsson <i>et al.</i> , 1998)	PCR for <i>sdrC</i> , <i>sdrD</i> , & <i>sdrE</i>
Bbp	Adhesin for bone sialoprotein	(Tung <i>et al.</i> , 2000)	PCR for <i>bbp</i>
EbpS	Adhesin for elastin	(Downer <i>et al.</i> , 2001; Park <i>et al.</i> , 1996)	PCR for <i>ebpS</i>
Map/Eap	MHC class II analog protein	(Boden & Flock, 1992; Jonsson <i>et al.</i> , 1995; McGavin <i>et al.</i> , 1993a; Palma <i>et al.</i> , 1999)	PCR for <i>map/eap</i>
<b>Toxins</b>			
TSST-1	Exotoxin with superantigen activity	Reviewed in (Bohach <i>et al.</i> , 1990)	PCR for <i>tst</i>
Enterotoxins A, B, C, D, E, G, H, I, & J	Exotoxins with superantigen activity	Reviewed in (Bohach <i>et al.</i> , 1990)	PCR for <i>sea</i> , <i>seb</i> , etc.
Exfoliative toxins A & B	Exotoxins with superantigen activity	Reviewed in (Bohach <i>et al.</i> , 1990)	PCR for <i>eta</i> & <i>etb</i>
Alpha toxin	Cytolytic pore-forming toxin	Reviewed in (Bhakdi & Tranum-Jensen, 1991)	lysis of rabbit erythrocytes
Beta toxin	Sphingomyelinase	Reviewed in (Arbuthnott, 1982)	hot-cold lysis of sheep erythrocytes
Delta toxin	Cytolytic toxin	Reviewed in (Arbuthnott, 1982)	Synergy of lysis with $\beta$ toxin producer
Panton-Valentine leukocidin	Bicomponent leukocidin	(Prevost <i>et al.</i> , 1995; Supersac <i>et al.</i> , 1993)	PCR for <i>pvl</i>
Gamma toxin	Bicomponent leukocidin	(Prevost <i>et al.</i> , 1995; Supersac <i>et al.</i> , 1993)	PCR for <i>hlg</i>
<b>Other factors</b>			
ica locus	Polysaccharide intercellular adhesion	(Cramton <i>et al.</i> , 1999)	PCR for <i>icaA</i>
coagulase	Binds prothrombin, activating conversion of fibrinogen to fibrin	(Hemker <i>et al.</i> , 1975; Kawabata <i>et al.</i> , 1985)	Coagulase test
Efb	Binds to fibrinogen	(Boden & Flock, 1994)	PCR for <i>efb</i>
V8 protease	Serine protease	(Drapeau <i>et al.</i> , 1972)	PCR for <i>V8</i>
agr subgroup	Global regulator	(Jarraud <i>et al.</i> , 2000; Ji <i>et al.</i> , 1997)	PCR for <i>agr</i> subgroup I, II, III and IV

### 8.2.2.1 PCR analysis.

Genomic DNA from *S. aureus* was extracted using the Wizard Genomic DNA purification kit, with the modification that lysostaphin 30µg/ml (Ambi) was added at the cell lysis step. *sea-i* and *tst* were amplified by multiplex PCR using the primers and conditions described elsewhere (McLauchlin *et al.*, 2000); *sej*, *eta* & *etb* were amplified using primers and conditions provided by G. O'Neill (personal communication). Primers and conditions used to amplify *efb*, *pvl* and *hlg* were as described in references (Boden Wastfelt & Flock, 1995) (*efb*), and (Lina *et al.*, 1999) (*pvl* and *hlg*). Primer design for the remaining determinants was based on gene sequences available from GenBank (Appendix 1). This was straightforward with the exception of *map/eap*, *agr* subgroup, and *sdrE* and *bbp*. There were three GenBank sequences available for *map*, (AJ243790, clinical isolate 7; AJ245439, Wood 46; AJ223806, Newman), and one for a gene termed *map/eap* (AJ290973, Newman). These were aligned and common forward and reverse primers designed. To distinguish between *agr* groups I, II, III and IV, use was made of sequence differences in or around *agrD*, the region encoding the autoinducing peptide. The forward primers for *agr* subgroups I and IV started nine bases upstream of the region encoding the peptide; primers for *agr* subgroup II and III started at the ninth or first base, respectively, of the region encoding the peptide. The reverse primers for all *agr* subgroups were within *agrC*.

Primer design for amplification of *sdrE* and *bbp* was complicated by the fact that these independently described genes are known to share significant homology (Tung *et al.*, 2000). Were these alleles of the same locus? The sequences for *sdrE* (GenBank accession number AJ005647) and *bbp* (Y18653) were blasted against the available genome sequence for two isolates undergoing sequencing at the Sanger Centre, Cambridge, UK using the website ([http://www.sanger.ac.uk/Projects/S\\_aureus/blast\\_server.shtml](http://www.sanger.ac.uk/Projects/S_aureus/blast_server.shtml)). These isolates were provided to the Sanger centre by Dr Nicholas Day and Dr Sharon Peacock

and will be called isolate 252 (methicillin-resistant isolate) and 476 (methicillin-sensitive) in the remainder of this work. Blast analysis demonstrated that *sdrE* and *bbp* co-localised to identical positions in the respective isolates. Isolate 252 shared 94% homology at the amino acid level with *bbp* and 79% with *sdrE*. In contrast, isolate 476 shared 80% homology with *bbp* and 93% with *sdrE*. The sequences for *sdrE* and *bbp* were aligned using Gene Jockey II (Biosoft, Cambridge, UK), and primers were designed to distinguish between what was assumed to be two alleles of a single gene by using a common forward primer but unique reverse primer. These primers were piloted using 50 randomly selected isolates. A clear distinction was seen between the primer pairs, with amplification of a single band product of the appropriate size for either *SdrE* (766bp), *bbp* (1054bp) or neither, and were used to evaluate the remaining isolates.

PCR amplifications were performed in a PTC-200 DNA engine (MJ Research, Waltham, Mass.) with *Taq* polymerase (Bioline). The final concentration of PCR reaction mixtures were 1x reaction buffer, variable magnesium concentration depending on optimization results (see Appendix 1), 100pmol of forward and reverse primers, 1 $\mu$ l of 1/5 dilution template DNA, 200 $\mu$ m 4dNTP mix, and 2.5 units of *Taq* polymerase. The PCR primers and cycling conditions not referred to elsewhere are shown in Appendix 1. Aliquots of the reaction mixtures were analyzed by 1% agarose gel electrophoresis. A positive control and negative control (reaction mixture minus DNA) was included in each PCR run (see Appendix 1).

#### **8.2.2.2 Phenotypic tests.**

Production of coagulase was evaluated using standard methodology (Collee *et al.*, 1996). Production of hemolysins was determined by streaking isolates onto a range of blood agar plates and incubated in air at 37°C for 18 hours. Isolates were considered positive for  $\alpha$ -toxin if hemolytic on 5% rabbit

blood agar, positive for  $\beta$ -toxin if haemolytic on 5% sheep blood agar following incubation at 37°C for 18 hours then 4°C for 30 minutes (hold-cold lysis), and positive for  $\delta$ -toxin if synergy was observed between the test isolate and a  $\beta$ -toxin producer on 3% washed sheep erythrocytes. Positive controls were NCTC5655 ( $\alpha$ -toxin positive), NCTC 7428 ( $\beta$ -toxin positive), and NCTC 9715 ( $\delta$ -toxin positive).

### 8.2.3 Analysis.

The isolates used in this study were chosen for two reasons. First, the case-control study design helps to avoid the potential confounding effect of comparing isolates from different geographical locations or time periods. Second, having defined the population structure of these isolates it was possible to adjust for the effects of clonality on associations between bacterial determinants and invasive disease. For example, a given determinant could become over-represented in the invasive group if it is linked to a 'true' virulence gene within a given lineage. However, analysis linked to interpretation of MLST results becomes model dependent. In view of this, in the primary analysis isolates were evaluated according to origin (nasal carriage isolate or invasive), and further sub-divided depending on whether the disease was hospital- or community-acquired. The second round of analysis used isolates grouped by lineage as defined by MLST results and analysis repeated for associations within each lineage. A lineage (or clonal complex) was defined for the purposes of this analysis as a group of organisms that had identical sequence at 5 or more of the 7 MLST loci examined. Further detail of the genetic structure of *S. aureus* can be found on the MLST website ([www.mlst.net](http://www.mlst.net)), and in Appendix 2.

Contingency tables were used to compare proportions between groups. To adjust for the 'hitchhiker effect', analyses were stratified by MLST-defined lineage using either the Mantel-Haenszel method or conditional logistic regression. Strains that were unrelated to any other at 5 of 7 loci were arbitrarily

grouped as a 'lineage' for the purpose of this stratification. No corrections were made for multiple comparisons. For the purpose of interpretation and discussion statistical significance was set at  $p=0.01$ , and for this reason conservative 99% confidence intervals are used throughout.

## 8.3 Results.

### 8.3.1 Univariate analysis.

Eight of the 33 determinants (24%) were significantly more common in invasive isolates (Table 8.2). The 8 determinants were as follows: 3 genes encoding cell wall-associated adhesins (*fnbA* encoding fibronectin binding protein A, *cna* encoding collagen binding protein, and *sdrE* which encodes a protein of unknown function); 4 genes encoding toxins (*sea* and *sej* encoding staphylococcal enterotoxins A and J, *eta* encoding exfoliative toxin A, and *hlg* encoding gamma toxin); and *icaA* (a marker for the *ica* operon), which is involved in biofilm production.

### 8.3.2 Adjusting for the effect of clonality.

That such a large proportion of the genes examined were associated with disease (albeit all positively) raises the question of whether the result can be explained by linkage disequilibrium between these genes and one or more 'true' virulence determinants elsewhere in the genome (the hitchhiker effect). In an attempt to adjust for this, the analysis was repeated, this time stratifying for clonality. The clonal structure of this collection of isolates has been defined by MLST ([www.mlst.net](http://www.mlst.net)); using this information and the Mantel-Haenszel method the prevalence of each gene was compared in the disease and carriage groups within but not between clonal complexes (Table 8.2, Fig. 8.1). Following this stratification all 8 genes remained positively associated with disease. *seg* was associated with disease on stratified analysis alone. Analysis was also repeated omitting what was by far the single largest clone, EMRSA-16 that contained 22 disease strains from a single group of hospitals but no carriage strains; without this clone *sea* was not associated with disease. In view of this, *sea* was not included as a virulence-associated gene in the remaining analysis. The other results were not affected, leaving 7 putative virulence determinants positively associated with disease across multiple *S. aureus* lineages.

**Table 8.2 Presence of putative virulence determinants in carriage and disease isolates – univariate analysis and analysis stratified by clonal complex.**

	Unstratified analysis				Stratified analysis	
	Carriage n=178	Invasive n=155	Odds ratio (99% CI)	p	Odds ratio * (99% CI)	p*
<b>Adhesins</b>						
<i>fnbA</i>	154 (87%)	152 (98%)	7.9 (1.7 to 77)	<b>0.0001</b>	12.0 (1.6 to 89)	<b>0.0001</b>
<i>clfA</i>	174 (98%)	155 (100%)	-	0.13	-	0.13
<i>clfB</i>	178 (100%)	155 (100%)	-	1	-	-
<i>cna</i>	57 (32%)	81 (52%)	2.3 (1.3 to 4.3)	<b>0.0002</b>	2.0 (1.1 to 3.7)	<b>0.003</b>
<i>spa</i>	160 (90%)	145 (94%)	1.6 (0.54 to 5.4)	0.24	1.7 (0.55 to 5.5)	0.20
<i>sdrC</i>	178 (100%)	156 (100%)	-	1	-	-
<i>sdrD</i>	73 (41%)	74 (48%)	1.3 (0.71 to 2.3)	0.27	1.9 (0.8 to 4.4)	0.05
<i>sdrE</i>	72 (40%)	87 (56%)	1.9 (1.0 to 3.4)	<b>0.006</b>	3.1 (1.4 to 6.7)	<b>0.0001</b>
<i>bbp</i>	76 (43%)	59 (38%)	0.82 (0.45 to 1.5)	0.43	0.74 (0.37 to 1.5)	0.27
<i>ebpS</i>	110 (62%)	105 (68%)	1.3 (0.70 to 2.4)	0.30	1.5 (0.65 to 3.4)	0.20
<i>map/eap</i>	165 (93%)	149 (96%)	2.0 (0.50 to 9.4)	0.24	1.7 (0.42 to 6.5)	0.34
<b>Toxins</b>						
<i>tst</i>	44 (25%)	46 (30%)	1.3 (0.68 to 2.6)	0.27	1.2 (0.46 to 2.9)	0.68
<i>sea</i>	30 (17%)	48 (32%)	2.3 (1.1 to 4.7)	<b>0.003</b>	2.6 (1.0 to 6.6)	<b>0.007</b>
<i>seb</i>	13 (7%)	14 (9%)	1.3 (0.41 to 3.9)	0.72	0.79 (0.22 to 2.8)	0.33
<i>sec</i>	20 (11%)	15 (10%)	0.84 (0.30 to 2.2)	0.72	0.85 (0.32 to 2.3)	0.66
<i>sed</i>	9 (5%)	8 (5%)	1.0 (0.24 to 4.2)	1	1.2 (0.34 to 4.4)	0.68
<i>see</i>	0	0	-	1	-	-
<i>seg</i>	113 (64%)	85 (55%)	0.69 (0.38 to 1.3)	0.12	0.42 (0.2 to 0.9)	<b>0.003</b>
<i>seh</i>	18 (10%)	24 (15%)	1.6 (0.65 to 4.1)	0.19	1.4 (0.63 to 3.3)	0.25
<i>sei</i>	106 (60%)	81 (52%)	0.73 (0.40 to 1.3)	0.18	0.50 (0.24 to 1.0)	0.01
<i>sej</i>	12 (7%)	38 (25%)	4.5 (1.8 to 13)	<b>&lt;0.001</b>	4.4 (1.7 to 11)	<b>&lt;0.0001</b>
<i>eta</i>	11 (6%)	34 (22%)	4.2 (1.6 to 13)	<b>&lt;0.001</b>	6.3 (2.0 to 20)	<b>&lt;0.0001</b>
<i>etb</i>	6 (3%)	0 (0%)	-	0.03	-	0.06
<i>pvl</i>	3 (2%)	6 (4%)	2.3 (0.31 to 27)	0.31	2.7 (0.38 to 19)	0.18
<i>hlg</i>	157 (89%)	150 (97%)	3.6 (0.97 to 19)	<b>0.01</b>	4.5 (1.0 to 19)	<b>0.004</b>
Alpha toxin	178 (100%)	153 (99%)	-	0.21	-	0.15
Beta toxin	160 (89%)	122 (79%)	0.45 (0.18 to 1.0)	0.02	0.43 (0.17 to 1.1)	0.01
Delta toxin	144 (80%)	106 (69%)	0.54 (0.27 to 1.1)	0.02	0.55 (0.27 to 1.1)	0.03
<b>Others</b>						
<i>ica</i>	136 (77%)	143 (92%)	3.5 (1.4 to 9.8)	<b>&lt;0.0001</b>	3.5 (1.4 to 9.1)	<b>0.0002</b>
<i>V8</i>	168 (94%)	149 (96%)	1.5 (0.34 to 7.5)	0.61	1.8 (0.42 to 7.9)	0.28
<i>efb</i>	105 (60%)	105 (68%)	1.5 (0.79 to 2.7)	0.11	1.7 (0.86 to 3.4)	0.03
Coagulase	175 (99%)	155 (100%)	-	1	-	0.30
<i>agr</i>						
subgroup I	65 (37%)	53 (34%)	0.91 (0.57 to 1.5)	0.73	0.43 (0.15 to 1.2)	0.10
subgroup II	43 (24%)	34 (22%)	0.89 (0.51 to 1.5)	0.70	3.3 (1.0 to 11)	0.04
subgroup III	65 (37%)	65 (42%)	1.3 (0.80 to 2.0)	0.31	0.78 (0.22 to 2.8)	0.70
subgroup IV	5 (3%)	2 (1%)	0.46 (0.04 to 2.8)	0.46	0.79 (0.13 to 14)	0.79

Odds ratio and p value after Mantel-Haenszel stratification for MLST-defined lineage. This was carried out to account for any influence of underlying clonality on the results.

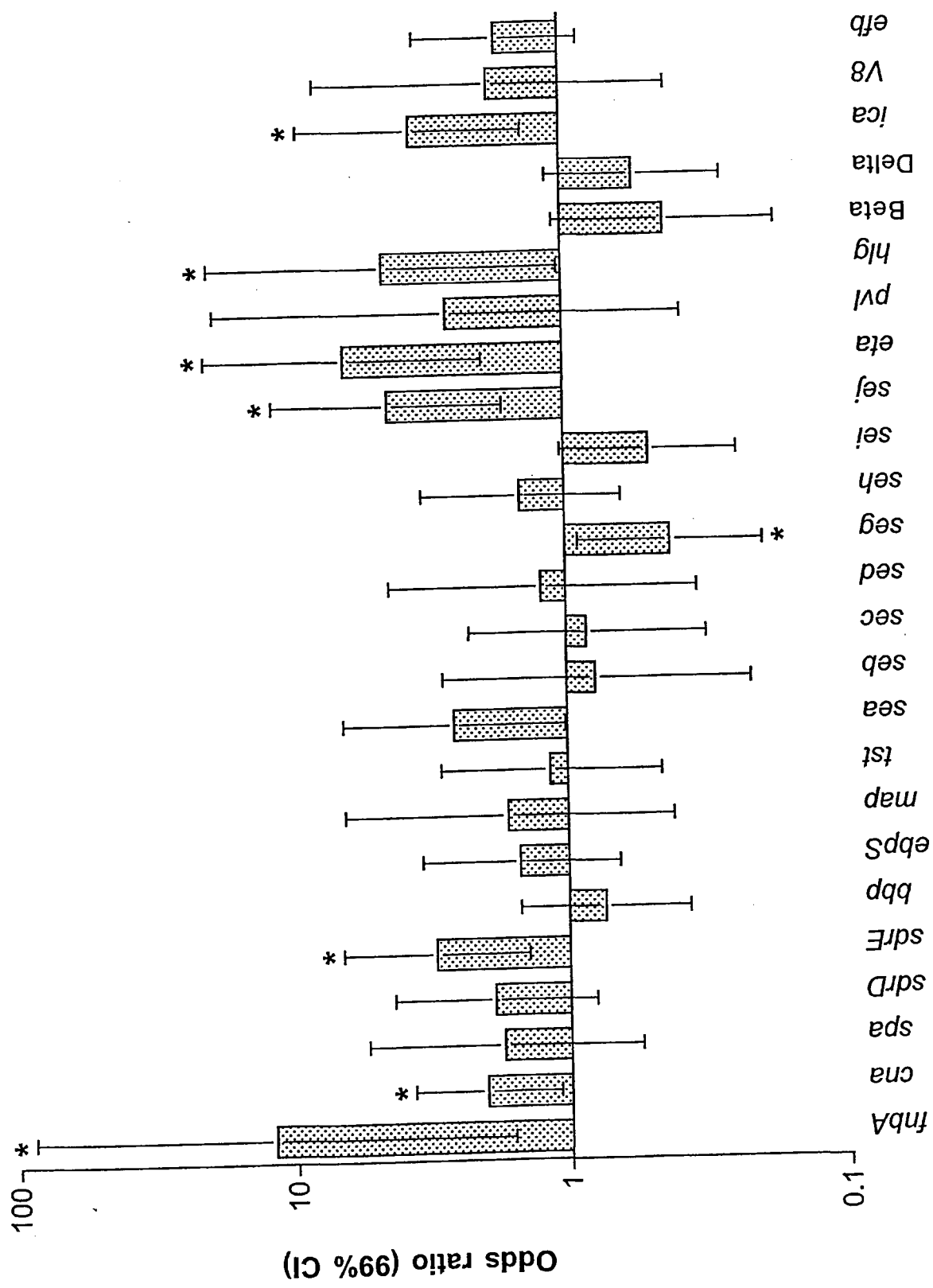


Figure 8.1 Presence or absence of putative virulence determinants: odds ratios for disease, adjusted for effects of clonality by Mantel-Haenszel stratification of MLST-defined lineage. Error bars denote 99% confidence intervals, and an asterix indicates a significant association with disease ( $p < 0.01$ ). Genes that were either ubiquitous (such as *clfA* and *clfB*) or very rare/absent (see and *etb*) are not shown.



### **8.3.3 Disease origin: community or hospital.**

The relative importance of the 33 bacterial factors in hospital versus community-acquired isolates was assessed by a comparison of carriage isolates and community-acquired disease alone. The presence of genes encoding six of the seven virulence-associated factors from the primary analysis (*fnbA*, *cna*, *sdrE*, *sej*, *eta* and *ica*) remained associated with disease once hospital-acquired strains were removed. Although *hlg* was numerically more common in the community-acquired disease group than the hospital-acquired group (98% vs 95%), the comparison with the carriage isolates (88%) was no longer statistically significant.

### **8.3.4 Multivariate analysis.**

#### **8.3.4.1 Linkage disequilibrium between virulence-associated genes.**

The possibility that identification of one or more of these 7 virulence-associated genes was actually the result of linkage disequilibrium within this group was examined. Logistic regression modelling of the relationship of the 7 factors to both disease and to each other suggested that four of the factors (*sej*, *fnbA*, *cna*, and *sdrE*) were both independent of each other and independently associated with disease. Two of the remaining three factors, *ica* and *hlg*, were in linkage disequilibrium with *fnbA*, though each contributed significantly to virulence after taking this association into account. *eta* was in linkage disequilibrium with *sej*, but also contributed independently to virulence. It was thus concluded that all 7 factors were associated with virulence.

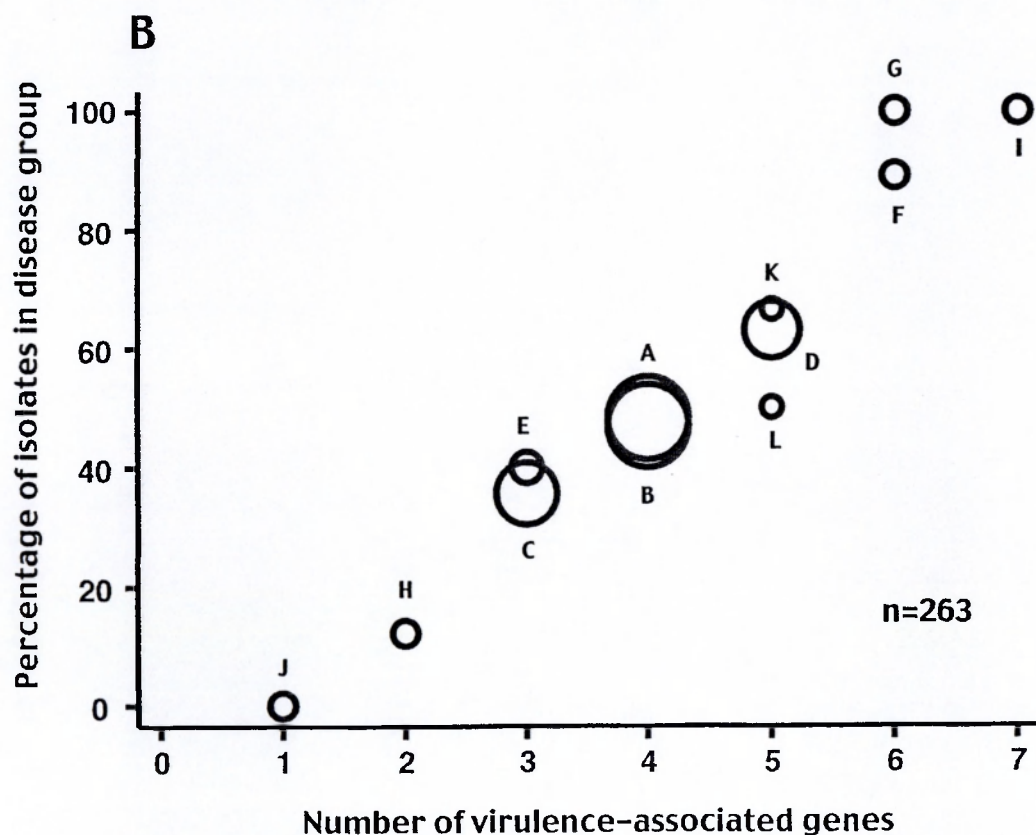
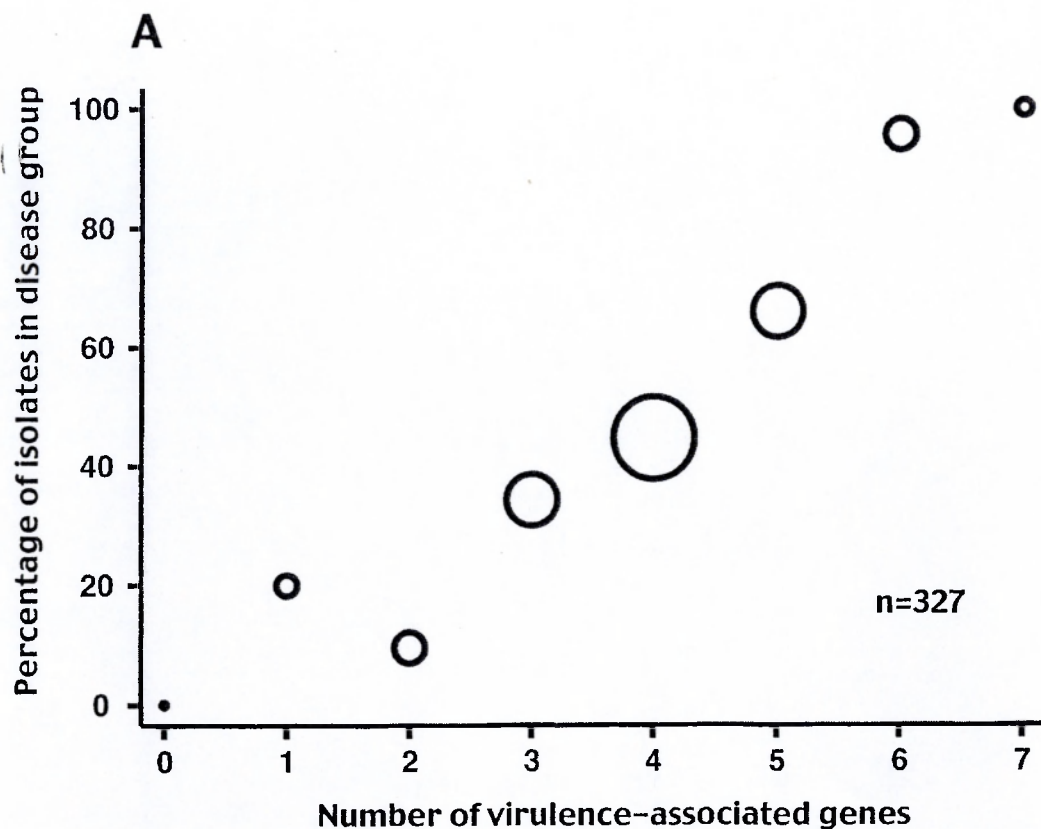
#### **8.3.4.2 Role of individual genes in disease.**

Although on univariate analysis the odds ratio for disease for *fnbA* was twice as high as any of the other virulence-associated genes, in the full multivariate model the odds ratios for disease for each gene fell between 2.2 (*eta*) and 3.3 (*fnbA*). This suggests that each of the 7 genes contributed

approximately equally to virulence, and that no single bacterial factor predominated as the major predictor of virulence.

#### **8.3.4.3 Cumulative effect of genes.**

There was a linear trend between the number of these 7 virulence determinants carried by particular isolates, and the proportion of isolates causing disease ( $p < 0.0001$ , Fig. 8.2A). This suggested that the effects of the factors on virulence were cumulative. The association between number of determinants and the odds of disease was log-linear, consistent with the logistic regression model in which there is a cumulative effect of each subsequent factor on virulence. None of the remaining 26 putative virulence determinants were associated with disease on univariate analysis, either separately or combined as a count of the number of determinants in each organism.



**Figure 8.2** Association between number of virulence-associated determinants and:  
 (A) proportion of isolates from cases of disease (rather than carriage). For example, 20% of isolates with one virulence-associated gene were from cases of disease and 80% were carriage strains.  
 (B) proportion of isolates with a given gene combination from cases of disease. Each combination is identified by a letter; for the actual gene complement refer to table 8.3. The 12 commonest combinations representing 80% of isolates are shown. The area of each circle is proportional to the number of isolates with that number of determinants.

8.3.5 Gene combinations and virulence.

Given that the effects of the seven genes on the odds of being a disease-causing strain are additive, analysis was performed to address whether particular gene combinations were either (i) more commonly found together in an organism than by chance, or (ii) more virulent.

In the study population there were 46 (of a possible 128) different combinations of the seven genes, with more than 80% of the isolates accounted for by 12 of these gene combinations (Table 8.3).

Table 8.3 Table of the 12 most common gene combinations.

Combination	<i>fnbA</i>	<i>cna</i>	<i>sdrE</i>	<i>sej</i>	<i>eta</i>	<i>hlg</i>	<i>ica</i>	N*	Number of isolates	Overall % of total	Cumulative % of total
A	1	0	1	0	0	1	1	4	64	19.6	19.6
B	1	1	0	0	0	1	1	4	64	19.6	39.1
C	1	0	0	0	0	1	1	3	39	11.9	51.1
D	1	1	1	0	0	1	1	5	35	10.7	61.8
E	1	0	1	0	0	1	0	3	10	3.1	64.8
F	1	0	1	1	1	1	1	6	9	2.8	67.6
G	1	1	0	1	1	1	1	6	8	2.4	70.0
H	1	0	0	0	0	1	0	2	8	2.4	72.5
I	1	1	1	1	1	1	1	7	7	2.1	74.6
J	0	0	0	0	0	1	0	1	7	2.1	76.8
K	1	0	1	1	0	1	1	5	6	1.8	78.6
L	1	0	1	0	1	1	1	5	6	1.8	80.4

\* Number of virulence-associated determinants within the gene combination (out of 7).

Actual and predicted frequencies of each gene combination were compared, assuming independent segregation of the genes. One common combination (denoted 'B' in Table 8.4) was over-represented in both carriage and disease (19.4% observed versus 11.7% predicted in carriage, and 18.6% versus 10% in disease); two virulent combinations (F & G, each with 6 virulence-associated genes) were over-represented in the disease group (5.2% versus

1.3%, and 5.2% versus 1.1%, respectively), and one less virulent combination (J, with 1 virulence-associated gene) was over-represented in the carriage group (4.1% versus 0.9%). No gene combinations were significantly under-represented compared with their predicted frequency. Are these discrepancies due to the effect of clonality, or do particular combinations of genes have either less or greater virulence than expected? None of the 12 most common combinations were either more or less likely to cause disease than others with the same number of virulence-associated genes, suggesting the effect was due to the clonal population structure (Fig. 8.2B).

**Table 8.4 Observed and predicted distribution of gene combinations.**

Gene combination	N*	Disease		Carriage	
		Observed %	Predicted %	Observed %	Predicted %
A	4	20.0	13.8	19.2	14.2
B	4	19.4	11.7	18.6	10.0
C	3	9.0	10.8	14.5	21.3
D	5	14.2	14.9	7.6	6.7
E	3	2.6	1.2	3.5	4.2
F	6	5.2	1.3	0.6	0.1
G	6	5.2	1.1	0.0	0.0
H	2	0.6	0.9	4.1	6.4
I	7	4.5	1.4	0.0	0.0
J	1	0.0	0.0	4.1	0.9
K	5	2.6	4.6	1.2	1.1
L	5	1.9	3.9	1.7	0.9

\* Number of virulence-associated determinants within the gene combination (out of 7). Where the observed frequency of a combination differs significantly from that expected, this is indicated by a '>' or '<' sign. Significance was determined by whether or not the exact binomial 99% confidence intervals around the observed percentage included the predicted percentage.

### 8.3.6 Effect of clonality on genes and gene combinations.

#### 8.3.6.1 Distribution of individual genes between lineages.

Contingency table analysis was used to examine whether individual genes were apparently randomly distributed between the major MLST-derived clonal complexes, or whether their distribution was significantly influenced by the underlying clonality of the population. *clfB*, *sdrC* and *see* were excluded from this analysis as they were present in all isolates. The genes fell into two distinct groups. The distribution of the enterotoxins, *tst*, the exfoliatins (*eta* and *etb*), beta and delta toxins, the variable *sdr* genes (*sdrD*, *sdrE* and *bbp*), *cna*, *ebpS* and *efb* within the population were all highly significantly related to clonal complex ( $p < 0.001$  in all cases). In contrast the distributions of *fnbA*, *clfA*, coagulase, *spa*, *map*, *pvl*, *hlg*, alpha toxin, *ica* and *V8* appeared to be unrelated to the underlying clonal structure ( $p > 0.1$  in all cases). Most of this second group are genes which are either very common or very rare, and it is possible that the absence of a significant association with clonal complex is due to lack of power, leading to a type II error. However the difference in the *p* value range between the two groups is striking, and even after removing from the second group those genes which are either present or absent in more than 95% of isolates *fnbA*, *spa*, *ica*, *map* and *hlg* remain apparently unrelated to underlying clonality (and 3 of these are 'virulence-associated' genes).

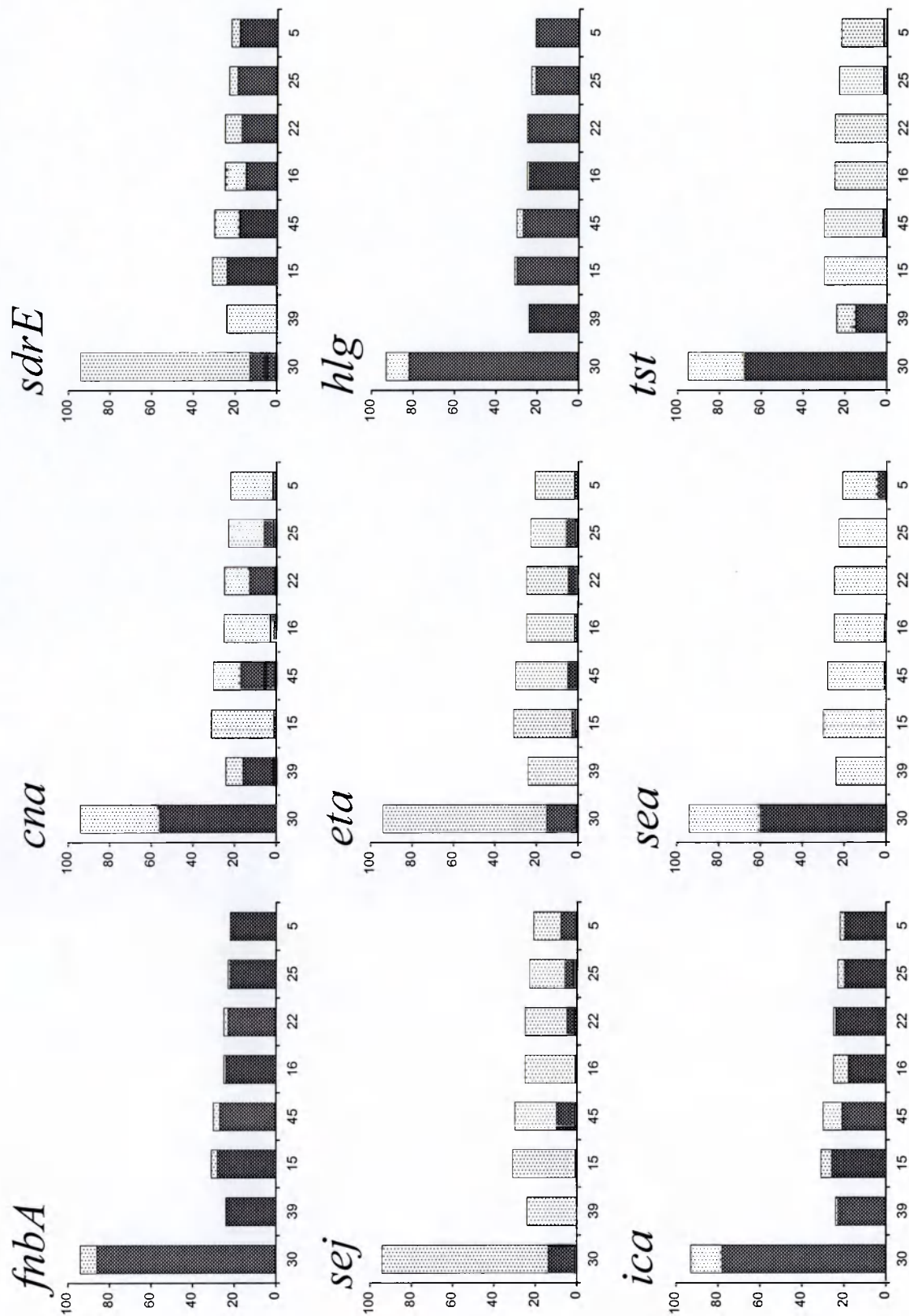
Although there is strong statistical evidence for an association with clonality for the first group of genes listed above, there is also evidence to suggest that horizontal transfer of genes plays an important role in determining the distribution of these genes. All the genes with a prevalence of more than 30% were found in isolates from all 11 major lineages. The exceptions to this diversity are *agr* subgroup I to IV, which were very tightly (but not completely) linked to the underlying clonal structure (Table 8.5).

**Table 8.5 Background clonality and *agr* subgroups \***

<b>Clonal complex</b>	<b><i>agr</i> I</b>	<b><i>agr</i> II</b>	<b><i>agr</i> III</b>	<b><i>agr</i> IV</b>	<b>Total</b>
CC30	2	2	<b>90</b> (96%)	0	94
CC39	0	0	<b>24</b> (100%)	0	24
CC15	0	<b>31</b> (100%)	0	0	31
CC45	<b>28</b> (93%)	0	1	1	30
CC22	<b>25</b> (100%)	0	0	0	25
CC25	<b>23</b> (100%)	0	0	0	23
CC5	3	<b>18</b> (86%)	0	0	21
CC1	3	0	<b>13</b> (81%)	0	16
CC8	<b>16</b> (100%)	0	0	0	16
CC12	0	<b>11</b> (100%)	0	0	11
CC51	1	3	1	<b>5</b> (50%)	10

\* Percentages represent % of organisms within that clonal complex. The population genetic structure of *S. aureus* can be found on the MLST website ([www.mlst.net](http://www.mlst.net)) and in Appendix 2.

The most clonal of all the genes characterised as either present or absent was *tst*, with 92% of positive isolates occurring in clonal complexes termed CC30 or CC39. These two clonal complexes are closely related (the only two which are), and probably derive from the same progenitor strain. One interpretation is that CC30 and CC39 represent a relatively recent but highly successful clonal expansion (perhaps fuelled by *tst*) which has had less time to diversify through horizontal transfer than other lineages. That other genes (*sea* and *bbp*) were also heavily polarised with respect to this complex lends support to this view. The distributions of the 7 virulence-related genes plus *sea* and *tst* are shown in Figure 8.3, along with the distribution of the *agr* subgroups; this selection includes genes which are chromosomally encoded (*ica*, *fnbA*, *sdrE*), and associated with plasmids (*sej*), bacteriophage (*sea*) and the SaPI 1 pathogenicity island (*tst*).



**Figure 8.3 Distribution of genes within the 8 largest MLST-defined clonal complexes (denoted by numbers along the x axis).** The overall height of each bar denotes the total number of isolates in the complex. The height of the dark-shaded area represents the number of isolates positive for the determinant for all the genes except *agr* subgroup. The population genetic structure of *S. aureus* can be found on the MLST website ([www.mlst.net](http://www.mlst.net)) and is shown in Appendix 2.



8.3.6.2 Distribution of gene combinations.

Underlying clonality had an important effect on the distribution of certain combinations of genes (linkage disequilibrium). The over-representation in both disease and carriage populations of virulence-associated gene combination B (containing *ica*, *fnbA*, *hlg* and *cna*, but lacking *sdrE*, *sej* and *eta*) (Table 8.4), is entirely because it was present in 51% of isolates from clonal complexes CC30 and CC39; its frequency in other clonal complexes was only 6%, not significantly different from its 'predicted' frequency. However, this clonality was not the explanation for the association of these genes with virulence; combination B was not itself associated with disease, and three of the four genes (*ica*, *fnbA*, and *hlg*) were significantly associated with disease within CC30/39, even after excluding those isolates with combination B. Despite the underlying clonality there was still considerable variation within clonal complexes and even within MLST sequence types. The 22 isolates of the nosocomial disease-causing EMRSA-16 clone, (a single MLST sequence type ST36), were represented by 10 different gene combinations, despite all being isolated within the same city over a two year period (see Table 8.6). ST36 was not unique in showing this diversity; the five most common combinations were all present in over half of the MLST-defined clonal complexes (data not shown), suggesting that horizontal transfer of genes between lineages is a relatively frequent occurrence.

**Table 8.6 Combinations of virulence-associated genes within the nosocomial infection-associated clone EMRSA-16 (sequence type 36).**

Combination	Frequency	Percent	<i>FnbA</i>	<i>cna</i>	<i>sdrE</i>	<i>sej</i>	<i>eta</i>	<i>hlg</i>	<i>ica</i>
B	7	32	1	1	0	0	0	1	1
G	4	18	1	1	0	1	1	1	1
C	3	14	1	0	0	0	0	1	1
M	2	9	1	0	0	0	0	0	0
N	1	5	1	1	0	0	0	1	0
O	1	5	1	1	1	1	1	1	0
P	1	5	1	1	0	0	1	1	1
Q	1	5	1	0	0	1	0	1	1
R	1	5	1	1	0	1	0	1	1
I	1	5	1	1	1	1	1	1	1

## **8.4 Discussion.**

### **8.4.1 Virulence-associated genes and clonality.**

These data represent a comprehensive analysis of virulence determinants in a large, natural population of *S. aureus* isolates of clearly defined clinical provenance. The finding that several bacterial factors were associated with invasive disease may directly reflect their involvement in the pathogenesis of severe disease. It is unlikely that one or more of the determinants have become over-represented due to the hitchhiker effect, as the positive associations remained despite stratification of the analysis by genetic lineage. This was demonstrated against a background in which many determinants were highly clonal, as shown in detail for several determinants.

### **8.4.2 Virulence-associated genes in the community and hospital setting.**

Host factors for *S. aureus* disease are likely to include a genetic predisposition via one or more susceptibility genes, and acquired factors such as the presence of intravenous devices, surgical wounds and other events that perturb normal host defences. It seems plausible to speculate that invasive disease occurs in two overlapping host populations. Individuals affected in the community (where the rate of disease is low) may represent those most genetically predisposed to *S. aureus* infection, while individuals infected in hospital (where the burden of disease is higher) may represent a much larger at risk group who may or may not carry susceptibility genes. Given this scenario, it is possible that the pattern or number of bacterial determinants associated with disease in the hospitalized host would differ from that seen in the community. However, there was little difference between strains associated with disease in the two settings, suggesting that bacterial factors play a role in causing disease even in the compromised host. Whether this is the case for truly immunocompromised individuals such as neutropenics and those with AIDS requires further study.

#### 8.4.3 Function of virulence-associated genes during the disease process.

This study of the presence of bacterial determinants in isolates associated with carriage and disease was assumption free with respect to their presumed function and biological effect during infection. Having identified 7 virulence-associated genes, the biological plausibility of these findings may now be considered. Fibronectin, the host ligand for the adhesin encoded by *fnbA*, is a ubiquitous host protein present in soluble form in the blood and in fibrillar form in cellular matrices, bacterial adhesion to which may be important during several steps of the disease process (Patti *et al.*, 1994a). Likewise, toxins that modulate the immune response through superantigen activity probably make a significant contribution to disease manifestations. However, it is also important to be somewhat circumspect when ascribing a given function to the pathogenic process, for the following reasons. First, many bacterial determinants are multi-functional (for example, fibronectin binding protein has recently been found to be an adhesin for fibrinogen) (Wann *et al.*, 2000), and the critical interaction of a given factor with the host may not yet have been defined. Second, the functions ascribed to a determinant by *in vitro* assays may not accurately reflect their behaviour *in vivo*. For example, it is possible that fibronectin binding protein is more important as an evader of the host immune response than as an adhesin *per se*. Bacteria in blood are likely to acquire a surface coat of fibronectin which could result in poor adherence to solid surfaces but may, for example, affect the efficiency of uptake by phagocytes. The finding that the presence of the *ica* operon is more commonly present in disease isolates is interesting. Biofilm is considered to be important in adherence to prosthetic material, and in persistence as bacteria become enmeshed in an exopolysaccharide that confers protection from the host immune system and the effects of antibiotics. It is possible that exopolysaccharide also confers protection in the absence of a foreign device or devitalised host tissue.

#### **8.4.4 The role of 'non-virulence-associated' and other determinants in disease.**

The study design enriched for the chances of finding virulence-associated determinants by pre-selecting those previously thought to be involved in disease, and excluding those with purely housekeeping functions. However, it is very unlikely that all of the genes responsible for disease manifestations have been identified in this study, an enterprise well suited to micro-array analysis. In addition, some of the 'non-virulence-associated' determinants as defined here may indeed play a role in pathogenesis, but because of their essential nature were not flagged as virulence-associated by the study design used here. For example, genes encoding the fibrinogen binding proteins ClfA and ClfB were ubiquitous, regardless of the origin of the strain.

#### **8.4.5 Virulence and allelic variation at a given locus.**

Studies of staphylococcal pathogenesis often focus on the presence or absence of a given determinant. The results above were analysed on the same basis. However, the nature of the methodology used here means that it is not possible to differentiate between the presence/absence of the entire gene, and the presence/absence of an allelic variant at a polymorphic locus. Southern hybridization may have been more sensitive than PCR in determining the presence or absence of a given gene. However, allelic variation may be important in defining virulence, and PCR may provide interesting clues. For example, *bbp* and *sdrE* appear to be allelic variants of each other, yet only *sdrE* was associated with virulence.

#### **8.4.6 Effect of virulence-associated genes on biological fitness.**

*S. aureus* usually behaves as a harmless human commensal as reflected by the large proportion of the healthy population who sometimes or usually carry this organism, the transition to disease representing the exception rather than the rule. So what is the relationship between virulence-associated genes and

bacterial fitness? The answer to this question is unknown, but is likely to be complex and could vary depending on the factor in question. For example, a determinant that confers the ability to invade the host may be associated with a fitness disadvantage since invasion could lead to bacterial death following antibiotic treatment or death of the host. Conversely, the presence of genes that enhance the ability to cause conditions such as superficial skin infection could lead to enhanced host-to-host transmission. A further consideration is that accessory genes that are important to disease pathogenesis but which are not critical to survival may impose a cost to fitness through additional gene replication and protein secretion if expressed during periods of carriage. It could be postulated, therefore, that the proportion of strains circulating in the community that carry virulence determinants, and the number of virulence-associated genes carried per strain, is a product of the interplay between rates of gene acquisition, the cost to biological fitness, and the rate of decay of strains causing human disease.

#### **8.4.7. Horizontal transfer of virulence-associated genes.**

The variation in virulence determinants in a given clone (as demonstrated for EMRSA-16), together with the widespread nature of combinations of virulence determinants, suggests that horizontal transfer of genes is a common event. The preservation of the associations between certain genes and virulence after stratification for bacterial lineage is in itself strong evidence for either loss or gain through horizontal transfer of genetic elements. The mechanism of transfer can be readily explained for 'mobile' determinants such as *sej* which is plasmid mediated (Zhang *et al.*, 1998). However, the majority of the virulence-associated genes are chromosomal, and it is not clear how such genes are being transmitted. In addition, not all 'mobile' elements appeared to undergo frequent horizontal transmission. An example of this is *tst* which resides on a pathogenicity island termed SaPI 1 (Lindsay *et al.*, 1998). This gene was very

common (>60%) in two closely related clonal complexes in this study (CC30 and CC39), but appeared either very infrequently or not at all in the remainder.

Although all the virulence-associated genes have plausible functional explanations for their involvement in disease genesis, and care has been taken to stratify for the major *S. aureus* lineages, the above analysis cannot exclude the possibility that one or more of these genes is a marker for a virulence gene which is either closely linked chromosomally or which co-segregates on the same mobile element.

### **8.5 Chapter summary.**

This study has demonstrated the variable presence of virulence-associated genes in natural populations of *S. aureus*, providing evidence that bacterial factors play a role in determining invasive disease in both community and hospital settings. The effect of these genes was cumulative, each independently multiplying the odds of disease. There was also evidence of considerable horizontal transfer of genes, on a background of clonality. This study also indicates that it may be an over-simplification to consider virulence in relation to the presence or absence of a given bacterial factor, since allelic variants of a polymorphic locus (such as *SdrE/bbp*) may make different contributions to the disease process.

## 9. Concluding comments.

This body of work started out examining the processes by which *S. aureus* interact<sup>s</sup> with human endothelial cells. The early studies proved fruitful and exciting, demonstrating that fibronectin-binding proteins are critical for bacterial adherence to, and uptake by endothelial cells; later studies developed largely as a result of these findings. This discussion will now consider the possible relevance of this work to interactions occurring in the human host.

The first question one might pose is a fundamental one, and that is, how do fibronectin-binding proteins contribute towards the ecological success of *S. aureus*? This question cannot be answered from the pool of current knowledge, and can only be addressed by developing postulates based largely on common sense. The first postulate is that FnBPs and the genes encoding them are maintained in the genome because they contribute to human carriage rather than to virulence. This is based on the premise that human carriage is essential for bacterial survival. Approximately 20% of the healthy population persistently carry *S. aureus*, while about 60% of individuals are intermittent carriers. The nose represents the ecological niche for this organism, and there are no known sustainable environmental reservoirs. Survival in this site is likely to involve the ability to adhere to the lining of the nose (keratinized squamous epithelium); to adapt to environmental changes such as cold, moisture and dryness; to evade the host immune defences (in particular IgA and anti-bacterial peptides); and the capability of transmission between hosts. Furthermore, adaptability and rapid response to change are likely to be vital to ecological success. In comparison with carriage, invasive disease is a rare event and appears to be of little biological benefit to the bacterium. Affected individuals do not have enhanced shedding unless infection involves the skin, they are usually treated with anti-staphylococcal antibiotics (where affordable), and infection may even result in death of the host. Thus, one could postulate that the presence and

pattern of bacterial determinants is driven primarily by the benefit they confer during colonisation in the nose.

Fibronectin-binding proteins are almost ubiquitously expressed by *S. aureus*, and as such are likely to fulfil an important role. While the maintenance of two *fnb* genes may be the result of linkage with other beneficial genes and the genome, it is also possible that two genes are in some way better than one. So what role could FnBPs have during colonisation? Possibilities include adherence to the squamous epithelium, and evading expulsion by the immune system by becoming coated with host proteins. Studies of the interaction between *S. aureus* FnBP and nasal epithelial cells, mucin and components of the mucosal immunity have not been reported in the literature. The functions served by FnBP may be necessary for many pathogens, since FnBPs are expressed by a range of bacterial species; defining what these are represents an important and clinically relevant area of research.

So why study determinants such as FnBP in relation to disease pathogenesis? One might speculate that the presence or absence of *fnb* genes or polymorphisms in the protein relate to biological fitness; however, once *in vivo* they could potentially make an important contribution to the outcome of an invasive event. The importance of studying this area is to gain an understanding of the interactions between the host and bacterium that may prove amenable to interference, such as vaccination against a bacterial epitope that is of central importance. Defining the involvement of FnBP in the interaction with endothelium could be an important starting point. However, there are many more questions to examine. For example, this study should be replicated using human dermal endothelial cells. These differ in surface receptors; for example, human dermal microvascular endothelium but not umbilical vein endothelium express CD36 (Swerlick *et al.*, 1992), a glycoprotein that may bind thrombospondin (Asch *et al.*, 1987) and collagen (Tandon *et al.*, 1989). They may, therefore, be a better representative of the microvasculature. The model



should also be expanded to examine factors such as the role of plasma proteins, the effect of shear flow and endothelial cell stimulation, and the mechanisms by which bacteria are taken up by the host cell. However, a more fundamental question that this is whether *S. aureus* is truly an intracellular pathogen. The benefits of being taken up by host non-professional phagocytes include evasion from the immune response and the effect of antibiotics. The reality at this present time is that *S. aureus* has not been demonstrated to be an intracellular pathogen *in vivo*. This does not mean that it is not, but rather that a difficult but important study of fatal human *S. aureus* disease needs to be conducted.

Studies based on *in vitro* systems offer an important opportunity to examine interactions between determinants at a cellular and molecular level, but are easy to criticise when trying to assess their relevance to human disease. However, the last study in this thesis is sufficiently robust in design to start to explore this question. Fibronectin-binding proteins were found to be virulence-associated, the effect of the determinants identified being cumulative. However, there are several potential contradictions that should be explored. The first is why carriage isolates were less likely to have a detectable *fnbA* in this study if, indeed, FnBPs are important in colonisation. Second, the study described in chapter 5 reported that all *S. aureus* isolates had at least one *fnb* gene by Southern dot blot, while the study of adhesins and toxins in chapter 8 found them to be very common but variable between carriage and invasive isolates. It is possible that this relates to vagaries in sampling, a possibility that could be tested by defining the population genetic structure of isolates examined in the first study and comparing this with those used in the second study. An alternative explanation lies with the difference in technique; the identification of a stretch of DNA by PCR relies on conserved sequence at the site of primer annealing. The difference in detection of *fnb* genes between carriage and disease isolates may be the result of genetic polymorphism which in turn influences

virulence potential, rather than the presence/absence of the entire gene. Further studies are now needed to explore this observation.

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**Appendix 1. PCR primers and condltions for amplification of *S. aureus* adhesin and toxin genes**

PCR product	Primer sequences	Positive control & product length	PCR conditions
<i>fnbA</i>	Forward, position 431 (accession J04151) 5'-CACAACCAGCAAATATAG-3' Reverse, position 1792 5'-CTGTGTGGTAATCAATGTC-3'	8325-4 1362bp	Mg 1.5 1 min 94°C 1 min 50°C 2 min 72°C
<i>clfA</i>	Forward, position 368 (accession Z18852) 5'-GTAGGTACGTTAATCGGTT-3' Reverse, position 1951 5'-CTCATCAGGTTGTTTCAGG-3'	Newman 1584bp	Mg 3.0 1 min 94°C 1 min 45°C 2 min 72°C
<i>clfB</i>	Forward, position 425 (accession AJ224764) 5'-TGCAAGATCAAACCTGTTCT-3' Reverse, position 1020 5'-TCGGTCTGTAAATAAAGGTA-3'	Newman 596bp	Mg 3.0 1 min 94°C 1 min 45°C 1 min 72°C
<i>cna</i>	Forward, position 1719 (accession M81736) 5'-AGTGGTTACTAATACTG-3' Reverse, position 3457 5'-CAGGATAGATTGGTTTA-3'	Phillips Variable*	Mg 1.5 1 min 94°C 1 min 55°C 2 min 72°C
<i>spa</i>	Forward, position 1 (accession J01786) 5'-TCGAAATAGCGTGATTTTGC-3' Reverse, position 1892 5'-GCACTGAGCAACAAAAGATG-3'	8325-4 1892bp	Mg 1.5 1 min 94°C 1 min 65°C 2 min 72°C
<i>sdrC</i>	Forward, position 481 (accession AJ005645) 5'-ACGACTATTAACCAAGAAC-3' Reverse, position 1040 5'-GTACTTGAAATAAGCGGTTG-3'	Newman 560bp	Mg 1.5 1 min 94°C 1 min 45°C 1 min 72°C
<i>sdrD</i>	Forward, position 361 (accession AJ005646) 5'-GGAAATAAAGTTGAAGTTTC-3' Reverse, position 860 5'-ACTTTGTCATCAACTGTAAT-3'	Newman 500bp	Mg 1.5 1 min 94°C 1 min 45°C 1 min 72°C
<i>sdrE</i>	Forward, position 650 (accession AJ005647) 5'-CAGTAAATGTGTCAAAAAGA-3' Reverse, position 1416 5'-TTGACTACCAGCTATATC-3'	Isolate 476 767bp	Mg 3 1 min 94°C 1 min 45°C 1.5 min 72°C
<i>bbp</i>	Forward, position 836 (accession Y18653) 5'-CAGTAAATGTGTCAAAAAGA-3' Reverse, position 1890 5'-TACACCCTGTTGAACTG-3'	Isolate 252 1055bp	Mg 3 1 min 94°C 1 min 45°C 1.5 min 72°C
<i>ebpS</i>	Forward, position 175 (accession U48826) 5'-CAATCGATAGACACAAATTC-3' Reverse, position 700 5'-CAGTTACATCATCATGTTTA-3'	Isolate 252 526bp	Mg 4 1 min 94°C 1 min 50°C 1 min 72°C
<i>map/eap</i>	Forward, position 203 (accession AJ243790), 255 (AJ290973), 257 (AJ245439) and 128 (AJ223806) 5'-TAACATTTAATAAGAATCAA-3' Reverse, position 1151, 1203, 1199 and 1076, respectively 5'-CCATTACTGCAATTGT-3'	Newman 943-949bp	Mg 3 1 min 94°C 1 min 45°C 1.5 min 72°C
<i>ica A</i>	Forward, position 2711 (accession AF086783) 5'-GATTATGTAATGTGCTTGGA-3' R Reverse, position 3480 5'-ACTACTGCTGCGTTAATAAT-3'	Isolate 252 770bp	Mg 4 1 min 94°C 1 min 50°C 1 min 72°C
<i>V8</i>	Forward, position 4 (accession Y00356) 5'-TTGTTCTTCGAAACTT-3' Reverse, position 1553 5'-GGCTTTGGCTTTATTG-3'	V8 1550bp	Mg 3 1 min 94°C 1 min 50°C 2 min 72°C
<i>agr</i> subgroup I	Forward, position 2405 (accession X52543) 5'-ATCGCAGCTTATAGTACTTGT-3' Reverse, position 3143 5'-CTTGATTACGTTTATATTCATC-3'	Isolate 2 739bp	Mg 3 1 min 94°C 1 min 50°C 1 min 72°C

<i>agr</i> subgroup II	Forward, position 642 (accession AF001782) 5'-AACGCTTGCAGCAGTTTATTT-3' Reverse, position 1332 5'-CGACATTATAAGTATTACAACA-3'	Isolate 19 691bp	Mg 3 1 min 94°C 1 min 50°C 1 min 72°C
<i>agr</i> subgroup III	Forward, position 642 (accession AF001783) 5'-TATATAAATTGTGATTTTTTATTG-3' Reverse, position 1353 5'-TTCTTTAAGAGTAAATTGAGAA-3'	Isolate 252 712bp	Mg 3 1 min 94°C 1 min 50°C 1 min 72°C
<i>agr</i> subgroup IV	Forward, position 1346 (accession AF88215) 5'-GTTGCTTCTTATAGTACATGTT-3' Reverse, position 2028 5'-CTTAAAAATATAGTGATTCCAATA-3'	Isolate 3049 683bp	Mg 3 1 min 94°C 1 min 50°C 1 min 72°C

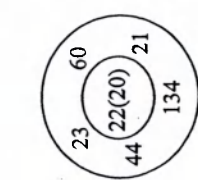
\*Variable product size depending on number of B repeats. Multiples of ~560 nucleotides

**Appendix 2. BURST diagram demonstrating population genetic structure of *S. aureus* as defined by multilocus sequence typing.**

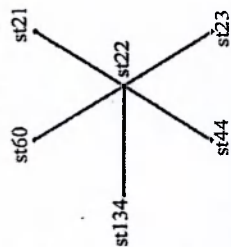
The figure (over) was kindly provided by Dr's Ed Feil, Nicholas Day and colleagues.

**Diagrammatic representation of clonal complexes.** Each number represents an MLST sequence type (ST). The central circle of each clonal complex contains the putative ancestral clone of each clonal complex. Single-locus variants (SLVs) of an ancestral clone lie within the next concentric circle, and double-locus variants within the outer circle. A solid straight line between two STs denotes a single-locus difference between them. Singletons are isolates possessing STs which differ from those of all other genotypes at > 2 loci. The clonal complexes are named according to the ST of the primary ancestral genotype, but with the prefix 'CC' (for clonal complex).

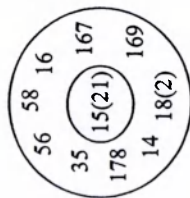




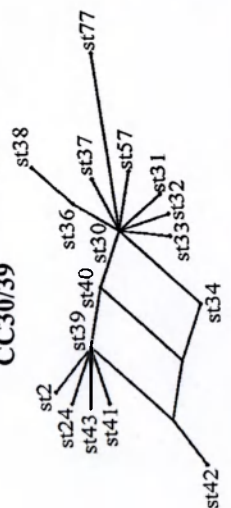
CC22



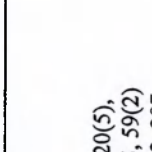
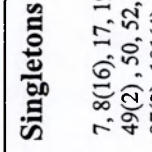
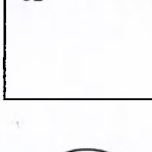
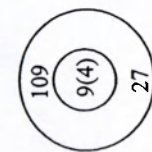
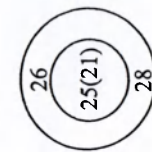
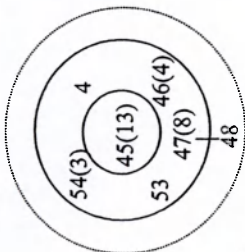
CC15



CC30/39



CC45



Singletons	
7, 8(16), 17, 19, 20(5),	
49(2), 50, 52, 55, 59(2)	
97(2), 101(4), 182, 207	
Minor groups	
123(3)	
121(5) — 51 — 29	69
6(2) — 5(19) — 11 3 — 1(11) — 188(3)	
145 — 10	12(10) — 13